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IMBALANCE BETWEEN NEUTROPHIL ELASTASE AND ELAFIN PROMOTES BREAST CANCER GROWTH AND PROGRESSION

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A DISSERTATION

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DEDICATION

This dissertation is dedicated to my parents:

Joseph A Caruso

and

Rosemary Barra Caruso

Their love, support, inspiration, and guidance have made everything possible.

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IMBALANCE BETWEEN NEUTROPHIL ELASTASE AND ELAFIN PROMOTES BREAST CANCER GROWTH AND PROGRESSION

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Abstract: Elafin, an endogenous serine protease inhibitor, is a critical component of the epithelial barrier against neutrophil elastase (NE) activity. The central hypothesis examined in this dissertation was that elafin has tumor suppressive properties in breast cancer. In support of this hypothesis, immunohistochemical (IHC) analysis revealed that elafin was downregulated in the majority of invasive breast tumors and a subset of pre-invasive ductal carcinoma *in situ* (DCIS) compared to elafin expression in the normal mammary epithelium. To understand the role of elafin in the mammary epithelium and the impetus for its downregulation during breast tumorigenesis, primary and immortalized human mammary epithelial cells (HMECs) were utilized as a model system. Elafin was highly expressed in G0-arrested HMECs, suggesting a previously unrecognized role for elafin in growth control. Stable knockdown (KD) of elafin compromised the ability of HMECs to maintain G0-arrest during long-term growth factor deprivation. This effect was reversed by re-expression of wild-type elafin but not elafin-M25G lacking protease inhibitory function, suggesting a role for deregulated protease activity. Elafin KD HMECs demonstrated enhanced sensitivity to NE-induced proliferation. Mechanistically, activation of the ERK signaling pathway downstream of toll-like receptor 4 (TLR4) was essential to the mitogenic effect of NE in this system. Compared to HMECs, the majority of breast cancer cell lines lack endogenous elafin expression. Adenoviral-mediated expression of elafin was utilized to evaluate the tumor suppressive properties of elafin in breast cancer cell lines. Rb-status was identified as the critical factor governing the anti-tumor effect of elafin in this system. In breast cancer cell lines expressing functional Rb, the expression of elafin triggered Rb-dependent cell cycle arrest. However, in breast cancer cell lines lacking functional Rb, elafin expression induced caspase-3 dependent apoptotic cell death. Elafin is a critical counterbalance against NE-activity. IHC analysis revealed that high levels of NE-expressing tumor-associated neutrophils (TAN) were associated with reduced

recurrence-free survival, high tumor grade, and triple-receptor negative breast cancer (TNBC). ERK-catalyzed phosphorylation of p90RSK (T359/S363) and Rb phosphorylation (S807/811) were significantly enriched in NE-positive breast tumors, suggesting that NE-induced ERK signaling and proliferation may be relevant to human breast cancer. The *in vivo* role of deregulated NE in breast tumorigenesis was examined in the C3(1)TAg mouse model of TNBC. Knockout of NE in C3(1)TAg mice significantly reduced tumor growth and proliferation. Elafin has tumor suppressive properties in the context of breast cancer and is a critical counterbalance against the growth promoting effect of NE *in vitro* and *in vivo*. Deregulated NE-activity is a viable therapeutic target in breast cancer.

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Recurrent Abbreviations

NE: Neutrophil elastase
PR3: Proteinase 3
HMEC: Human Mammary Epithelial Cells
G0: Quiescence
KD: Knockdown
TLR4: Toll-Like Receptor 4
Rb: Protein product of the Retinoblastoma gene (Rb1)
ERK: Extracellular Signal-Regulated Kinases
p90RSK: 90 kDa Ribosomal S6 Kinases
ECM: Extracellular Matrix
IHC: Immunohistochemistry
ER: Estrogen Receptor
PR: Progesterone Receptor
HER2: Human Epidermal Growth Factor Receptor 2
EGFR: Epidermal Growth Factor Receptor
PAR2: Protease Activated Receptor 2
MMP: Matrix Metalloproteinase
TEB: Terminal End Bud
TDLU: Terminal Ductal Lobular Unit
IDC: Invasive Ductal Carcinoma
DCIS: Ductal Carcinoma *In Situ*
ADH: Atypical Ductal Hyperplasia
ILC: Invasive Lobular Carcinoma
LCIS: Lobular Carcinoma *In Situ*
BM: Basement Membrane
BrdU: Bromodeoxyuridine
FACS: Fluorescence Assisted Cell Sorting
TNBC: Triple-Negative Breast Cancer
TAN: Tumor Associated Neutrophil
TAM: Tumor-Associated Macrophage
ROS: Reactive Oxygen Species
NSP: Neutrophil Serine Protease
ELISA: Enzyme Linked Immunosorbent Assay
IF: Immunofluorescence
RFS: Recurrence-Free Survival
OS: Overall Survival

Chapter 1: Introduction

Cancer is fundamentally a genetic disease. The accumulation of genetic and epigenetic alterations drives unrestrained proliferation and the invasive capacity of tumor cells (1). However, genomic derangement alone is insufficient for unabated tumor growth and progression. Post-mortem histological examination of adult tissues reveals a high frequency of occult tumors, held in stasis by a non-permissive microenvironment (2). Experimental evidence suggests that the ability of tumor cells to recruit and manipulate non-malignant cell types, including fibroblasts, endothelial cells, and leukocytes, governs their malignant growth potential (3-6). The interdependency of tumor cells and the surrounding stromal microenvironment in which they evolve (7) provides a strong rationale for the therapeutic targeting of essential crosstalk. The cellular constituents of the tumor microenvironment are not susceptible to the selective pressures driving resistance in tumor cells; therefore therapeutic modalities targeting microenvironmental factors may yield durable anti-tumor responses.

Extracellular proteases are powerful modifiers of stromal-epithelial crosstalk. Deregulated protease activity is implicated in the etiology (8) and progression of cancer (9). Consequently, endogenous protease inhibitors are critical to the maintenance of tissue homeostasis, while imbalance in protease-inhibitor stoichiometry is a significant component of protease deregulation during tumor progression (9, 10). Neutrophil elastase (NE) is a potent serine protease implicated in the pathogenesis of a wide variety of inflammatory diseases (11). High levels of NE, largely contributed by activated neutrophils in the tumor microenvironment, are prognostic of poor survival in human cancer (12). Elafin is a serine protease inhibitor produced by epithelial cells as a counterbalance against the deleterious effects of excessive NE activity (13). Permutations in elafin expression affect the integrity of the anti-protease shield and dramatically alter the pathogenesis of inflammatory disease (14-20). Elafin is downregulated in squamous cell carcinomas of the skin, head/neck, and esophagus (21, 22) as well as tumor-derived cell lines (23-27), suggesting that elafin loss is a feature of malignant progression.

The central hypothesis examined in this dissertation was that elafin has tumor suppressive properties in breast cancer. Based on the canonical function of elafin as an endogenous NE inhibitor, this hypothesis implicitly questions the tumor-promoting activity of deregulated NE in breast tumorigenesis. Chapter One is a review of three major topics relating to the research described in this dissertation: (1) the biology of the normal mammary gland, breast tumorigenesis, and the clinical management of breast cancer, (2) the physiological role of NE and the consequences of deregulated NE activity, and (3) the control of NE activity by elafin. In Chapter Two, the expression of elafin during breast cancer progression, the normal regulation of elafin in human mammary epithelial cells (HMECs), and the consequences of disequilibrium between elafin and NE were examined. In Chapter Three, the tumor suppressive properties of elafin following expression in breast cancer cell lines were investigated. In Chapter Four, NE expression in human breast tumors and the role of NE in a mouse model of breast cancer were explored. Chapter Five discusses the findings reported within this dissertation, the potential clinical application of the results presented, and the future directions for this research.

BREAST CANCER

Epidemiology of Breast Cancer

Breast cancer is the most frequently diagnosed malignancy and a leading cause of cancer mortality in women worldwide, accounting for 23-percent of female cancer diagnoses (1,380,000 cases; 2008) and 14-percent of female cancer fatalities (458,000 breast cancer deaths; 2008). Breast cancer disproportionately affects women in economically developed, westernized nations. The incidence of breast cancer is two- to three-fold higher in the developed countries of Western Europe and North America compared to the developing countries of Asia, South America, and Africa (28). Greater access to advanced mammographic screening technology exaggerates breast cancer incidence in developed nations and has contributed to the overall increase in breast cancer incidence observed since the late 1970s (29). However, the statistical anomaly created by breast cancer screening cannot completely rationalize the distribution and trends in breast cancer incidence over the last several decades. Lifestyle and environmental factors remain significantly associated with the increased incidence of breast cancer and observed geographical disparities. The increasing incidence of breast cancer within the population presents significant clinical, social, and economic challenges.

Despite rising incidence, breast cancer mortality has decreased in the United States and several other developed countries since the early 1990s. In 1991, the age-standardized breast cancer mortality rate in the United States was 32.7/100,000 per year; fifteen years later it had decreased to 23.4/100,000 per year (30). The overall five-year relative survival rate of women diagnosed with breast cancer between 2001 and 2007 was 90.0-percent, up from 75.1-percent for women diagnosed between 1975 and 1977 (31). Breast cancer survivorship has benefited from early detection by widespread mammographic screening, optimization of chemotherapeutic regimens, the standardized assessment of prognostic/predictive markers, and the introduction of targeted therapeutics. However, progress against breast cancer is unevenly distributed within the population. The age-standardized mortality of Caucasian women (28.3/100,000 per year) is significantly less than that of African American women (36.4/100,000 per year). Socioeconomic factors, access to screening, treatment

disparities, and fundamental genetic differences influence the unbalanced burden of breast cancer mortality (31, 32).

In the United States, one in eight women will be diagnosed and treated for breast cancer within her lifetime. Despite declining mortality rates over the last several decades (31), breast cancer remains a significant public health problem especially in economically developed countries.

Hereditary Susceptibility to Breast Cancer

Those affected by breast cancer or troubled by its prevalence are typically concerned with causality. However, no single factor can account for an individual's risk of developing breast cancer. Epidemiological evidence convincingly demonstrates that all common cancer types have a familial component (33, 34). Compared to the general population, the incidence of breast cancer is between two- and three-fold higher in the first-degree relatives of the cancer stricken (35). The aggregation of breast cancer within families is largely attributable to heritable factors (36, 37). In women diagnosed with breast cancer and treated by mastectomy, the rate of breast cancer incidence in the contralateral breast is two- to three-fold higher than the general population, suggesting that the majority of breast cancer occurs within genetically predisposed women. This association is seen regardless of the stage of the original tumor indicating that incidence of contralateral breast cancer was not a recurrence of the original tumor (38, 39). The study of cancer incidence in twins provides the most compelling evidence of a role for genetic factors in breast cancer susceptibility. In the monozygotic twins of women diagnosed with breast cancer, the incidence of breast cancer is three- to four-fold higher than in dizygotic twins or first-degree female relatives and six- to ten-fold higher than breast cancer incidence within the general population (35, 37).

Mutations in the highly penetrant breast cancer susceptibility genes, BRCA1 and BRCA2 (Table 1), are emblematic of hereditary breast cancer. Sub-populations with high frequencies of BRCA1 and BRCA2 mutations have been described, including Ashkenazi Jewish (40) and Bahamian (41) women. Women of African and Hispanic ancestry also have slightly higher frequencies of mutant BRCA1- and BRCA2-related breast cancers compared to woman of European or Asian descent (42, 43). In addition to BRCA1 and BRCA2 mutations, highly penetrate mutations in TP53 (Li-Fraumeni

syndrome), PTEN (Cowden syndrome), and STK11/LKB1 (Peutz-Jegher syndrome) are association with high breast cancer incidence (Table 1) (44-48). Together BRCA1, BRCA2, TP53, PTEN, and STK11/LKB1 mutations account for less than 25% of hereditary susceptibility to breast cancer (35, 49-51).

The majority of hereditary breast cancer susceptibility is believed to be polygenic, such that the genetic component of breast cancer risk is dependent on the combinatorial effect of several moderate and low penetrance gene variants. These alleles are not necessarily rare within the population, as is the case with mutations in BRCA1, BRCA2, TP53, PTEN, and STK11/LKB1 (49-51). The contribution of individual loci to cancer susceptibility is typically small, generally less than 1.3-fold, however the combinatorial effect can be significant.

Low penetrance polymorphisms at breast cancer susceptibility loci cannot be resolved by traditional genetic linkage studies, which relies on the mendelian inheritance of a rare disease-causing allele (52). Testing the association of polymorphisms in large cohorts of breast cancer patients versus controls is a powerful methodology to detect disease susceptibility loci. Pioneering association studies were limited by existing technology to the examination of candidate genes implicated in the etiology of breast cancer (53).

The sequencing of the human genome has cataloged over 10 million single nucleotide polymorphisms (SNPs) with allele frequencies greater than one-percent (54-56). The mapping of haplotypes, sets of SNPs that are statistically associated due to inheritances as a discrete unit (linkage disequilibrium blocks), allows the selection of a subset of genetic markers that define the majority of human genetic variability (56). These advances have facilitated the development of high throughput technologies capable of simultaneously assessing a great deal of common genetic diversity. Utilization of these technologies in association studies, referred to as genome-wide association studies (GWAS), has dramatically enhanced the capacity to detect disease susceptibility loci within the population. Large-scale genotyping remains an expensive and logistically challenging undertaking. GWAS generally employs a tiered design, beginning with a discovery phase that utilizes a relatively small cohort of cases and controls to identify potential susceptibility loci and followed by a validation phase utilizing much larger cohorts of case and controls (52).

High penetrance mutations in BRCA1 and BRCA2 account for a relatively small percentage of hereditary breast cancer susceptibility. GWAS studies have identified a significant number of low and moderate penetrance breast cancer susceptibility loci (Table 1). Individually these loci are associated with very modest increases in breast cancer susceptibility, however in combination their effect is pronounced. The majority of loci contributing to breast cancer susceptibility are located in intergenic and intronic chromosomal regions. The relevance of these regions is often unknown and functional significance is generally ascribed to the proximal gene based on the assumption that the majority of these loci fall within cis-regulatory elements (57).

Breast cancer is an extremely heterogeneous disease. Hereditary factors can influence susceptibility to particular biological subtypes of breast cancer. Women of African descent demonstrate increased susceptibility to aggressive, triple-receptor negative breast cancer (TNBC; estrogen receptor [ER] negative, progesterone receptor [PR] negative, and human epidermal growth factor receptor 2 [HER2] negative), suggesting the enrichment of TNBC susceptibility loci in this population (32, 58-60). BRCA1 and BRCA2 mutations are associated with the development of TNBC (61, 62). Knockout of Brca1 and Trp53 in the mouse mammary gland generates tumors with features of TNBC (63-65). Polymorphisms at the TERT-CLPTM1L (58), 19p13 (59), 16q12 (66), 5q11 (66), 11p15 (66), and 2q35 (66) loci are associated with the development of TNBC and some of these loci have been associated with women of African descent. ER-positive breast cancer susceptibility loci have also been identified in FGFR2 (67), TNRC9 (67), 8q24 (67), 2q35 (68), 16q12 (68) 9q31.2 (69), 5p12 (70), 10q26 (66), 3q24 (66), and 17q25 (66). The association of susceptibility loci with rare breast cancer subtypes is more difficult to ascertain due to the inability to generate adequately powered cohorts (66). Sufficient evidence exists that an individual's genotype plays a role in the genesis of specific breast cancer subtypes. Studies in larger cohorts are likely to identify more subtle associations between genetic variability and the genesis of breast cancer susceptibility.

Although cancer is a disease of somatically acquired genetic and epigenetic alterations, the underlying genotype of the individual also influences tumor biology. A recent study, utilized quantitative trait locus (eQTL: loci that regulate gene expression) based analysis to evaluate the contribution of germline eQTLs to tumor gene

expression. Using the cancer genome atlas (TCGA) database, cis-acting eQTLs were found to be responsible for 1.2% of the absolute variance in gene expression within breast tumors. Taking into account the effect of somatic genetic and epigenetic factors within tumors, the authors examined the effect of fifteen known breast cancer susceptibility loci, hypothesized to exist within cis-acting elements, on gene expression. Polymorphisms at 9q31, 8p24, and 9q31.2 were significantly associated with the expression level of ESR1, MYC, and KLF4 respectively (57). This study is notable in its ability to connect polymorphic loci with a functionally relevant change in gene expression.

The majority of breast cancer incidence likely occurs within a genetically susceptible subpopulation of women (36-39). Estimates suggest that about a third of the loci responsible for heritable breast cancer susceptibility have been identified, including high penetrance mutations (Table 1) (52, 53, 71-76). With rare exception, the contribution of individual polymorphisms to the etiology and progression of breast cancer is unknown. Building a comprehensive understanding of hereditary influences on breast cancer susceptibility is essential to understanding the etiology of breast cancer and more importantly identifying women at high risk of developing breast cancer who could benefit from the application of preventative medicine to this population.

Symbol	Name	Function	Penetrance	Disease
BRCA1 (77)	Breast cancer one, early onset	DNA repair: homologous recombination	High penetrance: mutant alleles also increase susceptibility to ovarian cancer	50-65% of women with deleterious mutation develop breast cancer by age 70 (78).
BRCA2 (79)	Breast cancer two, early onset	DNA repair: homologous recombination	High penetrance: mutant alleles also increase susceptibility to ovarian cancer	40-57% of women with deleterious mutation develop breast cancer by age 70 (78).
TP53 (80)	Tumor protein 53	Genome integrity, DNA damage response, apoptosis, cell cycle control, etc.	High penetrance: mutant alleles also increase susceptibility to leukemia, sarcomas, brain tumors etc.	TP53 mutations cause Li-Fraumeni syndrome. 49% of women with deleterious mutation develop breast cancer by age 60 (81).
PTEN (46)	Phosphatase and tensin homolog	Control of PI3K-signaling, which regulates proliferation, cell survival, cell growth, metabolism, etc.	High penetrance: mutant alleles increase susceptibility to thyroid, endometrial, colon, kidney cancer etc.	PTEN mutations cause Cowden Syndrome. 81% of women with deleterious mutation develop breast cancer by age 70 (82)
STK11 (LKB1) (83)	Serine-threonine kinase 11	Apoptosis and cell cycle regulation	High penetrance: mutant alleles also increase risk of gastrointestinal, pancreatic, ovarian, uterine, lung cancer, etc.	STK11 mutations cause Peutz-Jeghers syndrome. 32-54% of women with deleterious mutation develop breast cancer by age 60
CDH1 (84, 85)	E-cadherin	Cell adhesion	Moderate penetrance: mutant alleles also increase risk of colorectal and gastric cancer	Associated with the development of lobular carcinoma of the breast. 40-54% of women with deleterious mutation develop breast cancer by age 70.
PALB2 (86)	Partner and localizer of BRCA2	DNA repair, homologous recombination	Moderate penetrance	PALB2 mutations cause Fanconi anemia
ATM (87, 88)	Ataxia telangiectasia mutated	DNA damage checkpoint, double stranded breaks, DNA repair	Moderate penetrance	ATM mutations cause ataxia telangiectasia
CHEK2 (71, 89)	Checkpoint kinase 2	DNA damage checkpoint, double	Moderate penetrance	1100delC

		stranded breaks, DNA repair		
BRIP1 (90)	BRCA1 interacting protein c terminal helicase 1	DNA Repair: homologous recombination	Moderate penetrance	BRIP1 mutations causes Fanconia-anemia
RAD51C (76, 90)	RAD51 homolog C	DNA Repair: homologous recombination	Moderate penetrance	RAD51C mutations cause Fanconia-anemia like phenotype
XRCC2 (91)	X-ray repair complementing defective repair in chinese hamster cells 2	DNA Repair: homologous recombination	Moderate penetrance	
FGFR2 (74)	Fibroblast growth factor receptor 2	Receptor tyrosine kinase involved in mitogenic signaling, and differentiation	Low penetrance	Breast cancer associated variants cluster within intron two among punitive transcription factor binding sites.
TNRC9 (putative gene affected by 1612.1 variants) (68, 74, 92)	Trinucleotide-repeat-containing 9	DNA repair, gene transcription, poorly defined	Low penetrance	
MAP3K1 (putative gene affected by 5q11.2 variants) (74)	Mitogen-activated protein kinase kinase kinase one	Mitogenic signaling, component of the ERK signaling pathway	Low penetrance	
LSP1 (putative gene affected by 11p15.5 variants) (74)	Lymphocyte-specific protein one	F-actin binding protein, expressed by lymphocytes, neutrophils, and macrophages, may have a role in adhesion and motility of these cell types	Low penetrance	Cluster in intron 10
H19 (74)		Untranslated mRNA, regulated insulin-like growth factor two	Low penetrance: significance may be associated with linkage to LSP1 variants	Long non-coding RNA, maternally imprinted.
RAD51L1 (RAD51B) (putative gene affected by 14q24.1 variants) (93)	RAD51-like 1	DNA repair: homologous recombination	Low penetrance	
CASP8 (53, 94)	Caspase 8	Apoptosis	Low penetrance	D302H Shown to reduce breast cancer susceptibility.

ESR1 (putative gene affected by 6q25.1 variants) (69, 95)	Estrogen receptor alpha	Hormone signaling, mammary gland development, and breast cancer	Low penetrance	
MYC (putative gene affected by 8q24.21 variants)(96)	V-Myc myelocytomatosis viral oncogene homolog	Transcriptional regulation of cell proliferation, metabolism etc.	Low penetrance	
KLF4 (putative gene affected by 9q31.2 variants)(96)	Kruppel-like factor 4	Transcriptional regulation, stem cell marker	Low penetrance	
TERT (putative gene affected by 5p15 variants) (58)	Telomerase reverse transcriptase	Maintenance of telomere repeats	Low penetrance	Intron 4 polymorphism identified in women of African descent. Variant alleles associated with increased risk of developing ER-negative breast cancer
TGFβ1(94)	Transforming Growth Factor Beta 1	Pleiotropic cytokine with pro- and anti- tumor properties	Low penetrance	L10P
PTHLH (putative gene affected by 12p11 variants) (97)	Parathyroid hormone-like hormone isoform 1	Mammary gland and bone development	Low penetrance	
NRIP1 (putative gene affected by 21q21 variants) (69, 97)	Nuclear receptor-interacting protein 1	Transcriptional repressor of nuclear receptors including ER	Low penetrance	
COX11 (putative gene affected by 17q23.2 variants) (98)	Cytochrome c oxidase assembly homolog 11	Mitochondrial respiration, catalyzes electron transfer from cytochrome c to oxygen	Low penetrance	
SLC4A7/ NEK10 (putative genes affected by 3p24 variants) (98)	Solute carrier family 4, sodium bicarbonate cotransporter, member 7 Never-in mitosis related kinase 10	SLC4A7: extracellular pH NEK10: mitosis?	Low penetrance	

Table 1: Breast Cancer Susceptibility Genes.

Non-Heritable Breast Cancer Risk Factors

Non-heritable, environmental and physiological factors can also significantly affect the risk of breast cancer development. The mammary epithelium undergoes frequent and repeated remodeling events in the context of the female reproductive cycle and pregnancy (99). Despite the evolution of robust tumor suppressor pathways, dynamic tissues, such as the mammary gland, are inherently sensitive to transformation. Many environmental and physiological factors influence an individual susceptibility to breast cancer, including gender, age, estrogen exposure, childbearing, and lifestyle. The interplay between these factors and the underlying genetic susceptibility in the development of breast cancer is poorly defined.

Gender and Age

Breast cancer is 100-times more common in women than in men. Age is the greatest risk factors influencing breast cancer development in women. In European and North American countries, the cumulative incidence of breast cancer in women is 2.7-percent by 55 years of age, nearly doubling to 5-percent by 65 years of age, and increasing to 7.7 percent by 75 years of age (100). Following tumor initiation, the accrual of an adequate mutational spectrum capable of facilitating tumor growth/progression can take years or decades. The somatic mutation rate is believed to be the major factor limiting carcinogenesis. Under-recognized age-related changes in tissue architecture and the composition of the ECM may also play an important role in susceptibility to cancer by generating a microenvironment permissive of malignant cell expansion (101). Chronic low-grade inflammation and cytokine production are also associated with advanced age and significantly enhance the development of breast cancer (102). Rising life expectancy and aging populations in the United States, Western Europe, and elsewhere in the developed world will continue to increase the absolute incidence of breast cancer.

Estrogen Exposure

High-fidelity control of hormone levels is essential to the coordination of proliferation, differentiation, and apoptosis, processes that facilitate remodeling of the glandular architecture. Factors influencing hormone signaling are particularly important

determinants of breast cancer risk. Estrogen promotes growth and development of the mammary gland and also plays a central role in the etiology of breast cancer. The cumulative lifetime exposure of a woman to estrogen is consistently associated with the likelihood of breast cancer development (103). Early menarche (<12 years versus >16 years) and late menopause (>55 years versus <45 years) are significant breast cancer risk factors (104, 105). High levels of serum estrogen in post-menopausal women are associated with increased breast cancer incidence (106, 107). The pharmacological use of estrogen as a component of hormone replacement therapy (100) and to a lesser extent in contraceptives (108-110) increases breast cancer risk. Estrogen analogs increase the prevalence of breast cancer in heavily exposed populations. Estrogenic activity is a common property of industrial byproducts, herbicides, pesticides, and other synthetic products (111-114).

Childbearing

Pregnancy is associated with drastic changes in hormone signaling that can have confounding affects on breast cancer incidence. In the short term, high levels of serum estrogen can promote the progression of small pre-existing tumors and expand the pool of susceptible stem/progenitor cells, increasing breast cancer incidence in the years directly following pregnancy (104). In experimental models, pregnancy and lactation can induce changes in stromal ECM composition enhancing the invasive and metastatic capacity of breast tumor cells (115, 116) However, early-pregnancy (<20 versus >30) and multiple pregnancies greatly reduces long-term chances of developing breast cancer (104). Pregnancy is associated with high, sustained levels of circulating progesterone, inducing differentiation of mammary stem and progenitor cells, which is thought to underlie the long-term protective effect of pregnancy. Experimentally, carcinogen transformed mammary epithelial cells fail to form tumors in the stromal fat pad of uniparous rats, but readily form tumors in virgin rats. The factors responsible for the enhanced tumor suppressive properties of the post-partum mammary stroma are uncharacterized (117). Changes in the stroma and the stem/progenitor cell population within the mammary gland likely underlie long-term reductions in breast cancer risk following pregnancy. Lactation and prolonged breast-feeding are also known to reduce the risk of breast cancer, however the mechanism is undefined (118).

Obesity and the Western Lifestyle

Characterized especially by dietary overconsumption and a lack of physical activity, the western lifestyle encompasses a wide range of behavioral patterns and lifestyle choices known to promote several tumor types. Western women tend to give birth at relatively older ages and to fewer children, forgoing the protective benefit of early and repeated childbearing (119). A high fat diet and lack of physical activity are significant risk factors for breast cancer, especially in post-menopausal women (120). Resultant high body mass index (BMI) is strongly associated with breast cancer incidence (121-123). In post-menopausal women, the synthesis of estrogen is catalyzed in peripheral adipose tissue by the aromatase enzyme (CYP19). Dietary fat intervention studies demonstrate that reducing the percentage of calories consumed as fat significantly decreases circulating estrogen levels and breast cancer incidence, suggesting that increased estrogen signaling partially underlies breast cancer risk due to obesity (122, 124). In obese individuals, sub-clinical chronic inflammation is commonly observed in the visceral and subcutaneous adipose tissue. Dead adipocytes elicit an inflammatory response resulting in the recruitment of macrophages, which produce pro-inflammatory mediators known to promote tumor development and progression (125-128). In animal models, obesity induces significant inflammation of the mammary gland characterized by activation of the NF-κB pathway, elevated pro-inflammatory cytokine production, and enhanced aromatase expression (129). Alcohol consumption increases estrogen levels and breast cancer risk in a dose-dependent fashion (130). Perturbed hormonal homeostasis may be a significant mechanism by which the western lifestyle influences breast cancer incidence.

The western lifestyle may partially account for regional disparities in breast cancer incidence. Breast cancer incidence is more than three times higher in the United States than in Asian countries, including China, Japan, and the Philippines. Following migration to the United States, the incidence of breast cancer in Asian women from these countries shifts precipitously towards that of other Americans, reaching significance after only a decade; even when controlling for regional differences in heath care and surveillance. After two generations of residence in the United States, the relative risk of breast cancer is indistinguishable when comparing the descendants of Asian immigrants to other groups living in the United States (131). Increased breast

cancer incidence in recent immigrants and the overall trend of rising incidence among women in rapidly developing countries have been largely attributed to the westernization of these populations (132).

Breast cancer risk factors either increase the somatic mutation rate within the mammary epithelium and/or facilitate the aberrant growth and progression of breast cancer to clinical relevance. A comprehensive understanding of the environmental and hereditary factors influencing breast cancer susceptibility does not exist. The mechanisms underlying the effect of known risk breast cancer factors is largely absent or conjecture. Building a multivariate risk model for breast cancer could increase the efficiency of breast cancer screening programs and guide cancer-preventative strategies.

The Mammary Gland

The mammary gland is the defining characteristic of the class, Mammalia; evolutionarily designed, as an epidermal appendage, to provide for the rapid growth, development, and immunological defense of live-born offspring (133). The mammary gland is architecturally defined by ductal branching, a distinction that applies to several organs, including the kidney, salivary gland, vascular system, and lung. Most organs are patterned embryonically and maintain their basic structure throughout adulthood. Uniquely, the mammary gland experiences the majority of its growth and development after puberty and is subject to continual remodeling throughout the reproductive lifespan of the individual (134). Many of the same properties that make the mammary gland functional and responsive to the nutritional needs of offspring, also make it susceptible to tumorigenesis.

The labyrinthine molecular networks coordinating cell fate decisions, the proliferation of stem/progenitor cells, the initiation of context dependent differentiation programs, and maintenance of tissue homeostasis within the mammary gland are only partially understood. Essential paradigms of signaling are persistently observed, including the importance of stromal-epithelial interaction in the normal development and function of the mammary gland. Many of the developmental processes and molecular circuitry that shape the adult mammary gland, also play a role in promoting or inhibiting breast tumorigenesis and progression.

Murine Mammary Gland as a Model System

The availability of human breast tissue at discrete developmental stages is limited. The relatively few studies of human mammary gland development are restricted to histology, ultrastructure, and immunohistochemistry (135). *In vitro* culture systems have been developed that recapitulate some aspects of human mammary gland biology (136). However, mammary gland development and function is dependent on simultaneous interactions between a broad array of cell types and ECM elements, limiting the utility of *in vitro* systems. Therefore, the mouse has become the predominate model of mammary gland development. The transplantability of the adult mouse mammary gland makes it an ideal system for the examining the interaction between stromal and epithelial elements.

Despite 65-75 million years of divergent evolution, eighty-percent of human genes have direct orthologs within the mouse genome (137). Since their introduction in 1980s, transgenic (138, 139) and knockout (140-142) mouse models have significantly contributed to our understanding of the genetic and biological basis of mammary gland development and tumorigenesis. However, significant morphological, functional, and developmental differences exist between the mouse and human mammary gland, complicating the interpolation of observations made in the mouse to human physiology and disease (135, 143).

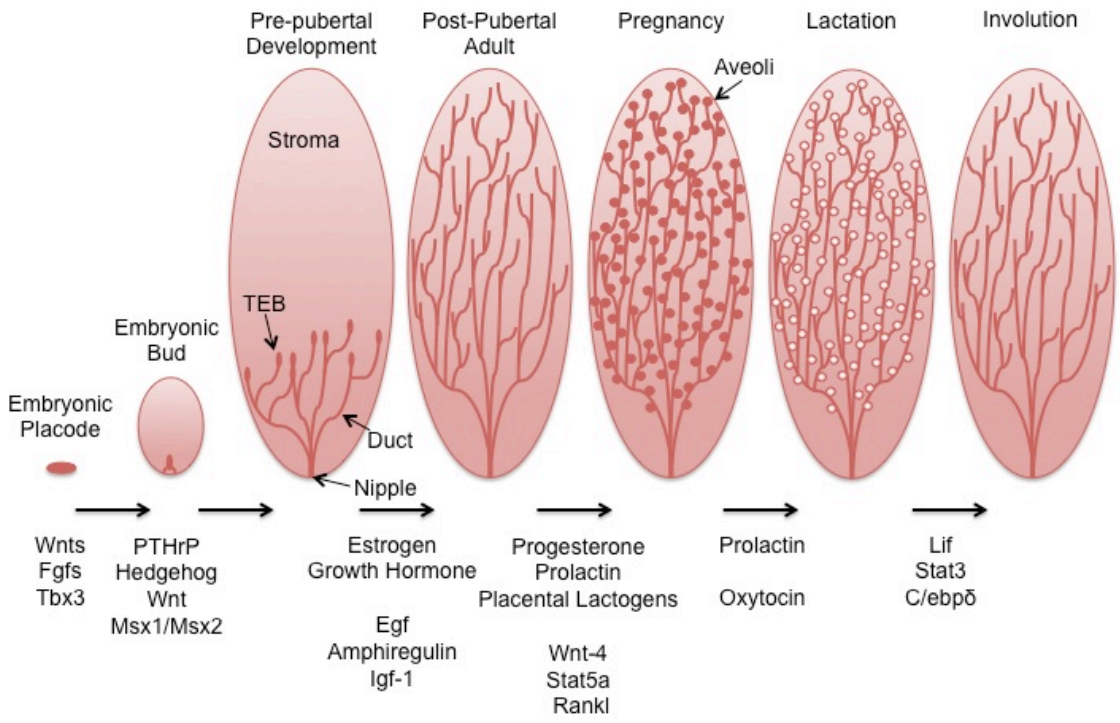

Figure 1: Critical Elements of the Signaling Network Regulating the Development of the Mouse Mammary Gland

The development of a ductal system capable of producing and delivering milk to offspring at parturition is a highly regulated process. The embryonic mouse mammary gland is formed by the invagination of epidermal cells from circular placodes into the dermal mesenchyme. Mammary cell fate decisions within the placode are dependent on Wnt and Fgf signaling as well as the Tbx3 transcription factor. Reciprocal paracrine exchange with the developing mesenchyme directs the development of the mammary bud; essential factors include Pthrp, hedgehog, and Wnt signaling as well as the Msx1/Msx2 transcription factors. At puberty, estrogen and GH drive branching morphogenesis through paracrine signaling intermediates including Egf, amphiregulin, and Igf1. Progesterone, prolactin, and placental lactogens drive lobuloalveolar differentiation through a series of paracrine intermediates, including Wnt-4, Stat5a, and Rankl. Upon parturition, high levels of prolactin and declining levels of progesterone, estrogen, and placental lactogens stimulate milk production. Oxytocin stimulates contraction of myoepithelial cells, triggering lactation. Following weaning, Lif-induced Stat3 synergizes with C/ebpδ to induce pro-apoptotic signaling and drive involution of the redundant epithelium.

Embryonic Mammary Gland Development

The initial stage of mammary gland development, as defined in the mouse, is the formation of bilateral milk lines; a multilayered epithelial ridge within the single layered embryonic epidermis. Nascent mammary epithelial cells then migrate to circular placodes, later forming the nipples, at regular intervals along the milk line (135, 144). Wnt/β-catenin signaling (145), fibroblast growth factor (Fgf) signaling (146), and the Tbx3 transcription factor (147, 148) are critical components of the molecular network directing early cell fate decisions at the mammary placode (Figure 1). Induction of Wnt/β-catenin signaling is among the earliest events at the mammary placode. Overexpression of the secreted Wnt inhibitor, Dickkopf 1, in the epidermis of mice abrogates mammary placode formation (145). Similarly, Fgf10, Fgfr2, and Tbx3 knockout mice cannot form embryonic placodes (146-148). Wnt/β-catenin (149, 150), Fgf (74, 151, 152), and Tbx3 (152, 153) pathways are commonly deregulated in breast cancer through overexpression, amplification, and in the case of some FGF-receptors, somatic mutation. Hyperactivity of these pathways been shown to enhance stem/progenitor cell populations and facilitate breast tumorigenesis in animal model (149, 150, 153).

The mammary bud is formed by the invagination of cells from the epidermal placode into the underlying dermal mesenchyme. The development of the mammary bud is dependent on reciprocal, paracrine exchange with the mammary mesenchyme (99, 154). The influence of mesenchymal signals on glandular epithelial development is very significant, such that transplantation of embryonic mammary epithelial cells into salivary gland mesenchyme results in the generation of salivary gland-like structures (155). The embryonic mammary mesenchyme even has the capacity to induce differentiation of mammary carcinoma cells (6). The importance of cellular context to mammary epithelial cells has also been demonstrated *in vitro* using three-dimensional culture on reconstituted basement membrane. Three-dimensional culture systems are capable of inducing functional differentiation of mammary epithelial cells and normalizing tumor cells, largely through epigenetic reprogramming (136, 156).

Several factors have been shown to drive mammary bud development, including Wnt/β-catenin signaling (157), parathyroid hormone related peptide (Pthrp) (158, 159), hedgehog signaling (160-162), and the Msx1/Msx2 transcription factors (163, 164)

(Figure 1). Pthrp signaling exemplifies paradigmatic stromal-epithelial crosstalk during mammary gland development. The mammary epithelial bud expresses Pthrp, while mesenchymal cells express the Pthrp receptor (Pth1r). Pthrp/Pth1r signaling drives the specialization of mesenchymal cells, the formation of the nipple, and suppresses hair follicle formation around the nipple. Pthrp has no direct effect on the developing mammary epithelium. Instead, Pthrp stimulates the expression of secreted mesenchymal factors essential to epithelial growth and differentiation, including bone morphogenetic protein 4 (Bmp4), neuregulin 3, and Fgf10. Pthrp knockout mice are incapable of maintaining the mammary bud (158, 159, 164, 165).

The hedgehog pathway also plays a prominent role in the development of the mammary bud. Similar to Pthrp, hedgehog ligands are exclusively expressed by the mammary epithelium. Haploinsufficiency of the Patched-1 hedgehog receptor (Ptc-1) is sufficient to disrupt early mammary gland development; evidenced by ductal hyperplasia and dysplasia. Transplantation of Ptc-1 +/- epithelium into a Ptc-1 +/+ stromal compartment completely reverses the phenotype, further evidencing the importance of stromal signaling during mammary epithelial development through paracrine feedback (161, 162). The hedgehog transcriptional affecter, Gli2, is exclusively expressed within the mammary mesenchyme. Knockout of Gli2 completely inhibits formation of the epithelial bud, corroborating the importance of stromal hedgehog signaling (160).

Embryonic mammary gland development is heavily dependent on reciprocal paracrine exchange between the epithelial and stromal compartments. The wnt, hedgehog, and Pthrp pathways are commonly deregulated in breast cancer and have pleiotropic roles in the tumor initiation and progression (166-172).

Post-Pubertal Mammary Gland Development

Relatively little is known about the molecular mechanisms governing embryonic ductal outgrowth and formation of the rudimentary mammary ductal tree evident at birth. Although embryonic mammary epithelial cells express hormone receptors in the presence of maternal hormones, knockout of hormone receptor fails to diminish the embryonic ductal outgrowth, indicating that embryonic ductal morphogenesis is hormone independent (144). The ductal tree remains virtually growth arrested

(undergoing only isometric growth) from birth until circulating ovarian hormones trigger profound expansion at puberty.

The importance of steroid and peptide hormones in the regulation of adolescent mammary gland development is well known. In rats, ovariectomy prevents ductal outgrowth of the mammary gland; administration of estrogen rescues this deficiency (173). Hypophysectomized (pituitary gland removal) rats require growth hormone (GH) and estrogen supplementation for mammary gland development (174). Estrogen receptor alpha (ERα) knockout mice and GH receptor knockout mice are incapable of ductal morphogenesis (175-178). Knockout of the estrogen receptor beta (ERβ), progesterone receptor (PR), and prolactin receptor do not significantly affect branching morphogenesis (178).

ERα is primarily expressed in the epithelial compartment of the mammary gland, displaying a heterogeneous distribution (179, 180). Despite the role of estrogen in the proliferation of the mammary gland, ERα expressing epithelial cells rarely proliferate due to TGF-β blockade (181). Transplantation of ERα -/- mammary epithelial cells into an ERα +/+ stroma does not rescue the ability of these cells to undergo branching morphogenesis (182). Conditional ERα knockout within the mammary epithelium is sufficient to abrogate ductal morphogenesis (175). Therefore, only epithelial ERα activity is required for mammary gland development. ERα-/- mammary epithelial cells, transplanted with ERα +/+ cells, are capable of proliferation and contribute to ductal elongation, evidencing a predominately paracrine mechanism of mammary gland development upon estrogen stimulation (182).

Amphiregulin is strongly induced by estrogen signaling in the mammary gland and is required for ductal morphogenesis (Figure 1). The protease ADAM17 is required for amphiregulin shedding and is also essential for ductal morphogenesis (183). Knockout and transplantation studies demonstrate that the epidermal growth factor receptor (EGFR), for which amphiregulin is a ligand, is active in the stromal compartment and required for ductal morphogenesis. Exogenous EGFR-ligands rescue ductal branching in ovariectomized mice (184). Downstream factors expressed in response to EGFR activation in the stromal compartment, include matrix metalloproteinase 2 (MMP-2) and FGFs. Knockout of MMP2 or conditional knockout of FGFR2 within the mammary epithelium impairs branching morphogenesis (185, 186).

The available evidence suggests that a paracrine feedback loop exists between ERα-induced, epithelial amphiregulin and stromal EGFR-induced paracrine factors (183, 187).

In contrast to ERα, the GH receptor (GHR) is expressed in the mammary stroma. Knockout of GHR in the mammary epithelium does not impair mammary gland development, however complete knockout of the GH signaling in both the epithelial and stromal compartments completely abolishes ductal outgrowth (188-191). GH drives the expression of insulin-like growth factor 1 (IGF-1) in stromal cells (Figure 1). Complete knockout of IGF-1 or knockout of its receptor in the mammary epithelium prevents ductal morphogenesis. Administration of IGF-1 to hypophysectomized rats or GHR-knockout mice restores growth of the mammary ductal tree, however administration of GH and estrogen to IGF-1 knockout mice does not rescue ductal morphogenesis (174, 190, 192).

Hormone signaling is essential to the development and function of the mammary gland. Estrogen and GH play a central role in the ductal elongation and morphogenesis. The human mammary gland undergoes constant remodeling during the reproductive lifespan of an individual. Hormone levels fluctuate during the menstrual cycle. Depending on the stage of the menstrual cycle, between four- and twenty-percent of luminal mammary epithelium expresses the ERα (180, 193). High estrogen levels drive mammary epithelial proliferation during the menstrual cycle. The proliferative fraction of mammary epithelial cells reaches 35% during the luteal phase, evidencing the scale of cyclic epithelial turnover. As estrogen levels decline, equivalent levels of apoptotic cell death counterbalanced the increased cellularity of the mammary gland (194, 195). The high levels of proliferation stimulated by estrogen underlie breast cancer risk associated with the lifetime exposure to estrogen.

In addition to its critical role in normal mammary gland development, estrogen is essential to tumor growth and progression. Three-quarters of breast tumors express ERα and are largely dependent on estrogenic stimulation for growth and progression. Tumors lacking ERα may also arise from estrogen responsive lesions in the breast (196, 197).

Mammary Gland Architecture

The mouse mammary ductal tree grows from terminal end buds (TEBs), multi-layered, club-shaped structures situated at the tips of the immature ducts. TEBs contain mammary stem/progenitor cells, which differentiate into two, morphologically unique cell layers, an outer layer of cap cells and interior body cells. Proliferation within the TEBs forces invasion into the mammary stroma and ductal elongation. The mammary tree fans out through the stroma by bifurcation of the TEBs and lateral side branching from ducts. Branching continues until the TEBs reach the peripheral extent of the mammary fat pad, at which point TEBs arrest and disappear largely due to the TGF-β signaling (198, 199). Apoptosis clears the cells within the duct forming a central lumen, a process dependent on upregulation of the pro-apoptotic regulator, Bim (200). Mature mammary ducts are bi-layered tubes; a single layer of columnar epithelial cells line a central lumen (luminal cells), supported by a layer of contractile myoepithelial cells (basal cells) (201). The cap cells of the TEB become the basal layer of the duct and body cells become the luminal layer.

The human mammary gland develops similarly as a branched network of ducts and has a comparable epithelial composition. The most significant morphological difference is the presence of lobules at the end of each duct, resembling an aggregate of grapes on a stem. This function unit of the human mammary gland is referred to as the terminal ductal lobular units (TDLU). Lobules within the TDLU consist of spherical alveoli are separated by connective tissue (Figure 2). The alveoli within the TDLU become the milk producing units of the mammary gland during lactation.

The epithelium of the TDLU is responsive to ovarian hormone stimulation and contains a pool of proliferative mammary stem/progenitor cells. These properties are retained during the reproductive lifespan of the individual, facilitating the structural remodeling of the mammary epithelium during the reproductive cycle and pregnancy. Consequently, the TDLU is the major site of breast tumorigenesis. The mouse equivalent of the TDLU is the lobuloalveolar unit. In contrast to the TDLU, lobuloalveolar units form at the terminus of regularly space tertiary side branches, which sprout from the mature ducts in response to cyclic exposure to ovarian hormones (143, 202).

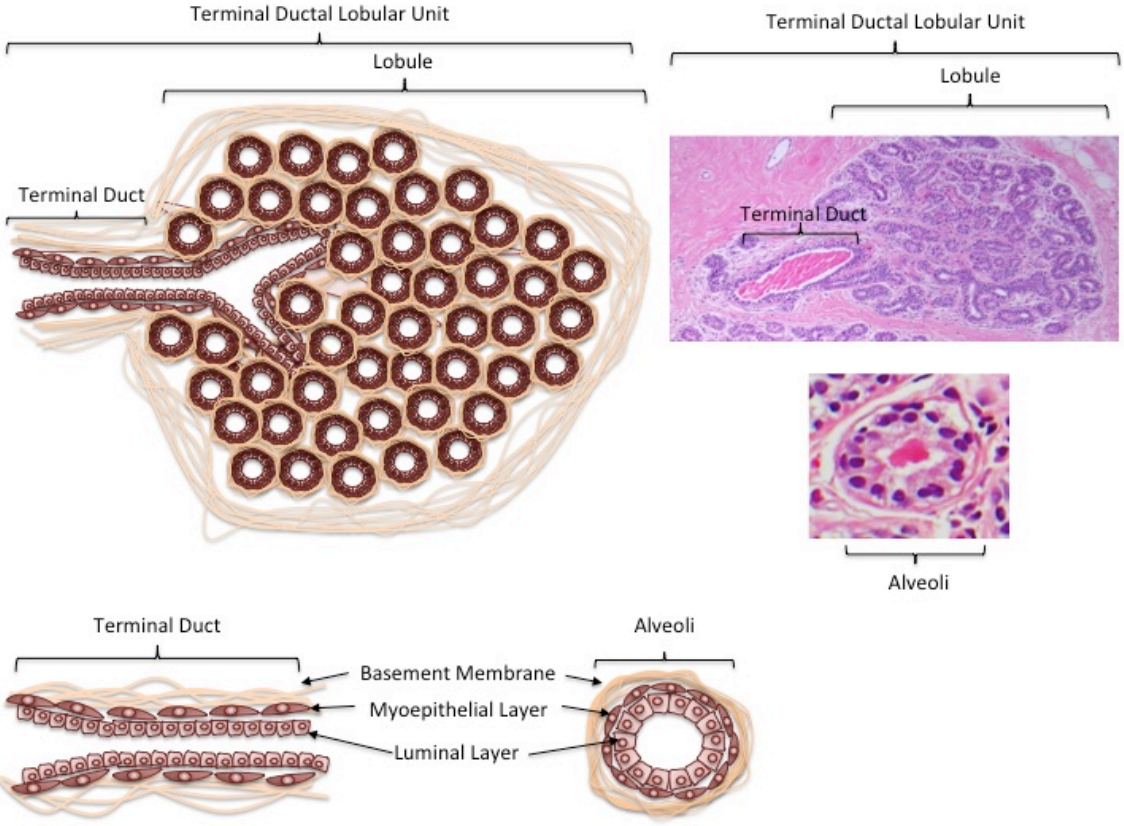

Figure 2: Terminal Ductal Lobular Unit

The function unit of the adult human mammary gland is TDLU, which is composed of the terminal duct and the lobule. The lobule is tightly bound collection of alveoli, which are hollow spheroids connected to the terminal duct. A single layer of epithelial cells lines the lumen of the alveoli and ducts. A layer of contractile myoepithelial cells and a layer of basement membrane surround the luminal epithelial layer. During lactation, functional differentiation of the alveoli produces the constituents of milk. Contraction of the myoepithelial layer forces the milk through the ducts and out the nipple. The epithelium of the TDLU is responsive to ovarian hormone stimulation and contains a pool of proliferative mammary stem/progenitor cells, making it the preferential site of breast tumorigenesis.

The Mammary Stroma

The mammary parenchyma exists within a complex stromal compartment. The mammary stroma consists of ECM and various cell types, including adipocytes, fibroblasts, endothelial cells, myofibroblasts, and immune/inflammatory cells (203). The mammary stroma is not simply a structural unit. Stromal derived signals are essential to the growth, differentiation, and maintenance of the mammary gland. Transplantation of mammary epithelial cells between mouse strains demonstrates that strain-specific differences in branching patterns are dependent on stromal factors (204). Mammographically dense breast tissue is a significant risk factor for human breast cancer development, suggesting that breast composition may influence tumorigenesis. (105, 205, 206).

Mechanical properties associated with ECM density and signaling molecules secreted by stromal cells significantly influence mammary epithelial cell behavior. A critical difference between the mouse and human mammary glands is the composition of the stromal compartment. Within the human breast, the mammary epithelium is embedded within a thick layer of highly collagenous connective tissue, separating it from the adipose tissue within the breast. Comparatively small amounts of fibrous connective tissue separate the mouse mammary epithelium from the adipose tissue (143, 202). Experimentally, the mammary fat pad of immunocompromised mice must be pre-injected with human fibroblasts and ECM to generate a stromal microenvironment conducive to the transplantation of human mammary epithelial tissue (207).

The mammary epithelium is surrounded by a layer of basement membrane (BM). Physical connection between mammary epithelial cells and BM components is critical for the control of apical-basal polarity, epithelial architecture, proliferation, and differentiation (208-210). Critical components of the mammary BM include collagen type IV, laminin 1, 5, 10, and 11, fibronectin, and heparin sulfate proteoglycans. The composition and thickness of the BM varies relative to the mammary duct (211). The composition and distribution of the BM significantly influences elongation, branching, and lumen clearance (101, 212). Transmembrane proteoglycans, integrins and non-integrin receptors expressed by the mammary epithelium detect the BM composition, dictating response. The cellular constituents of the mammary gland express several

dimeric integrin receptors capable of specifically detecting ECM elements, including collagen (α1β1 and α2β1), laminin (α3β1 and α6β4), and fibronectin (α5β1 and α5β3). Knockout of α2-integrin reduces ductal branching, (213), collagen receptor discoidin domain receptor 1 (DDR1) knockout mice are incapable of ductal elongation (208), and β1-integrin knockout disrupts alveologenesis (214-216). These results demonstrate the essential role of interactions between the BM and epithelium to the development and function of the mammary gland.

Inflammatory signaling networks and effectors of the inflammatory processes are important components of mammary gland elongation and development. Macrophages are recruited by the developing TEB, contributing angiogenic factors, proteases, cytokines, and growth factors. Colony-stimulating factor 1 (CSF-1) knockout mice are deficient in mature macrophages and demonstrate impaired branching morphogenesis (217). MMPs are critical to stromal remodeling and are known to dramatically affect invasion, elongation, and branching patterns. MMP-3 and MMP-2 are of particular importance in branching morphogenesis; MMP-2 knockout mice are incapable of invasion at TEBs, while MMP-3 knockout mice are deficient in lateral side branching (186). MMPs have a critical role in invasion and metastasis in breast cancer. Overexpression of MMPs, results in disruption of normal mammary gland development, hyperplasia, and in some cases is sufficient to initiate tumorigenesis (8, 218-221).

The composition of the mammary stroma is essential to the growth, differentiation, and function of the mammary gland (99, 154). Tumor progression is highly dependent on the ability of the tumor epithelium to recruit and manipulate non-malignant stromal cell types, including fibroblasts, endothelial cells, and leukocytes (3-6).

Pregnancy, Lactation, and Involution

During pregnancy, the mammary gland undergoes a dramatic increase in size and structural complexity, facilitating lactation at parturition. The fundamental component of the lactating mammary gland is the alveolus, a hollow spheroid with a central lumen. A single layer of secretory epithelial cells lines the alveoli lumen, which is surrounded by myoepithelial cells (222). Progesterone and prolactin drive lobuloalveolar differentiation. Progesterone receptor (PR) knockout mice demonstrate impaired

alveolar development (223, 224). PR (existing as two isoforms PR-A and PR-B) is expressed in both the stromal and epithelial compartments. In chimeric mammary glands, PR-/- epithelial cells are able to contribute to alveologenesis in the presence of PR+/+ epithelial cells, evidencing a paracrine mechanism of action. Essential downstream secreted factors include Wnt-4 and Rankl (receptor activated by NF-κB ligand) (Figure 1) (224, 225). Overexpression of Rankl rescues lobuloalveloar development in PR-knockout mice (226, 227). Prolactin receptor (PLR) knockout mice are similarly deficient in lobuloalveolar differentiation and lactation (228). Following prolactin stimulation, PLR and Jak2 associate resulting in the activation of Stat5a (Figure 1). Knockout of Stat5a displays the same defect in lobuloaveolar differentiation seen in prolactin knockout mice (229-231). Stat5a and PLR are both capable of enhancing Rankl transcription. Rankl knockout, Rankl knockout, or IκB knockin mice demonstrate failure of lobuloaveolar proliferation and differentiation, evidencing a role for the NF-κB pathway in growth and differentiation of the secretory epithelium (232). Rankl is frequently deregulated during breast cancer progression and plays a critical role in metastasis to the bone (233-235).

At parturition, functional differentiation of the secretory epithelium and lactation occurs in response to declining estrogen and progesterone levels, loss of placental lactogens, and high levels of prolactin. During lactation, the secretory epithelium produces the constituents of milk, secreting them into the alveoli lumen. Stimulated by oxytocin, myoepithelial cells contract, forcing the milk through progressively larger ducts, and eventually the nipple (Figure 1) (222, 236, 237).

Following weaning, the milk-producing epithelium involutes through extensive apoptosis returning the mammary ductal tree to its original size and complexity (238). Involution of the mammary epithelium is dependent on Stat3, induced by leukemia inhibitory factor (LIF) (239). Knockout of Stat3 inhibits apoptosis of the secretory mammary epithelium following forced weaning (240, 241). Stat3 induces the expression of c/ebpδ, synergy between these two transcription factors potently induces pro-apoptotic gene expression (Figure 1), including Bak, Igfbp5, and death receptor ligands (tnfα, FasL, Trail, and Tweak) (242-244). Stat3 activation also inhibits Akt survival signals by upregulating PI(3)Kinase regulatory subunits p55 α and p50 α (245). As the redundant mammary epithelial undergoes apoptosis, the activation of inflammatory

pathways leads to expression of MMPs and serine proteases. Protease activation enhances apoptosis through the removal of matrix attachment and is necessary for mammary gland remodeling. Apoptotic cells are cleared by recruited immune cells and adipoctyes replace the epithelium (246).

The mammary gland is capable of repeated cycles of alveologenesis, lactation, and involution following subsequent pregnancies, facilitated by stem and progenitor cell maintained within the TDLU (247). Ultimately, at the conclusion of the reproductive lifespan of the individual, menopause, the levels of ovarian hormones decline and the functional elements of the mammary gland atrophy. In mammals, significant efficiency in energy utilization is derived from tightly coupling mammary gland development and function with reproduction (154, 248, 249).

The high levels of proliferation throughout the reproductive lifespan and the essential role of stem/progenitor cells in mammary gland remodeling are believed to sensitize the mammary gland to tumorigenesis. The majority of breast tumors occur in post-menopausal women. Sustained estrogen stimulation due to the extra-ovarian synthesis of estrogen is critical to the development of breast tumors in post-menopausal women (120).

Breast Tumor Histopathology

Histological examination of breast tumors reveals a great deal of heterogeneity. Greater than 90% of breast cancers are carcinomas, however several sarcomas are known to occur within the breast. Invasive ductal carcinoma (IDC) of the breast, arising from the terminal duct, is the most common form of breast cancer, accounting for 65-80% of cases. Ductal carcinoma *in situ* (DCIS), a non-invasive accumulation of neoplastic cells within the duct, is generally accepted to be a precursor of invasive breast cancer (250, 251). Invasive lobular carcinoma (ILC) of the breast, arising from the lobules, accounts for approximately 8-14% of breast tumors, while 3-6% of breast tumors display mixed lobular/ductal histology. Despite significant molecular difference between IDC and ILC, clinical outcomes are similar in multivariate analysis. Loss of e-cadherin expression appears to be a critical factor in the development of ILC. Lobular carcinoma *in situ* (LCIS) is not a necessarily a precursor lesion, however it is predictive of subsequent development of breast cancer (either IDC or ILC) (252, 253). Several

additional subtypes of breast cancer commonly observed based on histological features. Tubular carcinoma (4%), invasive cribriform carcinoma, and mucionous carcinoma (2%) generally have a good relative prognosis. Medullary carcinoma (2%) has an intermediate prognosis. Metaplastic carcinoma (<1%), signet-ring carcinoma (<1%), inflammatory carcinoma (<1%), and lipid rich carcinoma (<1%) have a relatively poor prognosis. The molecular characteristics and origins of the rare histological subtypes are currently not well understood and the majority of research attention is directed at IDC given its prevalence.

Mammary Stem and Progenitor Cells

The regenerative capacity of the mammary gland is contingent on a stem and progenitor cell population capable of continuously replenishing the mammary epithelium. Mammary stem and progenitor cells are a prominent topic of research investigation not only because of their role in mammary morphogenesis and tissue homeostasis, but also because they provide a rich set of targets for transformation.

Murine Mammary Stem and Progenitor Cells

The existence of stem cells in the mouse mammary gland was established by pioneering studies in which normal mammary gland fragments or disassociated mammary epithelial cells were transplanted into the cleared mammary fat pad of syngeneic mice (254). A complete and functional mammary gland can be generated using mammary epithelial cells from any portion of the donor ductal tree, regardless of donor age or parity (255, 256). Reconstitution of the mammary gland following serial transplantation evidences a capacity for self-renewal, a critical property of stem cells. However, unlike neoplastic tissue, the regenerative capacity of normal mammary epithelial cells in serial transplantation studies is limited (between five and seven iterations) (257, 258). Transplantation of retrovirally labeled mammary epithelial cells demonstrated that a single cell can repopulate the entire mammary ductal tree indicating multipotency (the ability to differentiate into multiple cell types), another critical characteristic of stem cells (259). Serial transplantation of cells at limiting dilutions and examination of mammary repopulating efficiency is the gold standard methodology for verifying stem cell properties and establishing their frequency (260, 261).

Long-term label retaining cells can be identified by bromodeoxyuridine (BrdU) or ^{3}H-thymidine pulse and chase experiments, suggestive of infrequent and asymmetric divisions, providing additional evidence of a stem cell population with the adult mammary gland (261, 262). Ultrastructure and histological studies identified several populations of undifferentiated cell types within the mouse mammary glands, which have been hypothesized to be stem and progenitor cells (255).

Isolation of stem cell populations is difficult due to their rarity and failure to maintain their phenotype in culture. Using cell surface markers and fluorescence-assisted cell sorting several impressive studies have overcome the barriers to mammary stem cell purification from disassociated mouse mammary glands. The authors of one study were able to generate a functional mammary gland at limiting dilution with cells expressing CD24 (heat stable antigen) and high levels of CD49f (α6-integrin) (260). Similarly, another group was able to reconstitute the mammary gland using a single cell expressing CD24 and high levels of CD29 (β1-integrin) (261). In both studies, serial transplantation into the cleared mammary fat pad of secondary recipient mice reconstituted the mammary gland, demonstrating the hallmark capacity for self-renewal. The ability of the reconstituted mammary glands to form milk producing lobuloalveolar units during pregnancy confirms the ability of the identified mammary stem cells to differentiate into the critical lineages comprising the mammary gland (260).

Bi-potent and lineage restricted progenitors have been identified in the mouse mammary gland suggesting a hierarchical organization of mammary stem and progenitor cells. Committed luminal progenitors can be purified based on high expression of CD61 (β3-integrin), low levels of CD133 (prominin-1), and low levels of Sca-1. Differentiation into mature luminal cells is accompanied by loss of CD61, increased CD133, and increased Sca-1 (263-265). The number of intermediates, their nature, and the relationships between them are not fully understood. Characterization of individual progenitor cells is challenging. Cell surface markers are an imperfect means to consistently segregate pure populations of progenitors. Specific cell surface markers have not been identified for myoepithelial progenitor. Plasticity likely exist within the mammary stem and progenitor cell hierarchy further complicating their purification and characterization ex vivo.

Lineage Tracing of Murine Stem and Progenitor Cells

Lineage tracing is an important tool for the examination of stem and progenitor cell hierarchy *in vivo*. Using the K14 promoter to drive YFP expression, a mutipotent progenitor can be identified in the embryonic mouse mammary gland. However, postnatal lineage tracing using inducible K14-YFP reporter mice find a K14 expressing, lineage restricted subpopulation of progenitor cells capable of generating cells of the myoepitelial, but not luminal lineage cells. Inducible-YFP expression under the K5 and Lgr5 promoters, confirms the existence of unipotent myoepithelial progenitor cells in the mature mouse mammary gland. Using inducible-K8-YFP reporter mice, a subpopulation of progenitor cells were identified that can give rise to the luminal lineage alone. These results argue that in the adult mammary gland, luminal and myoepithelial lineages are derived independently from distinct lineage-restricted progenitor cell populations. Interestingly, transplantation of the unipotent myoepithelilal (but not luminal) progenitor alone into a cleared mammary fat pad forces them to become multipotent and reconstitute both the luminal and myoepithelilal compartments. Transplantation of myoepithelilal progenitors with luminal progenitors maintains their unipotent nature (247). This indicates that plasticity exists between the myoepithelial progenitor and mammary stem cell populations and possibly explains the discordant results of lineage tracing and transplantation experiments.

Lineage-tracing using WAP-Cre and Rosa26LacZ reporter mice has also been used to identify a long-lived sub-population of parity-identified mammary epithelial cells (PI-MECs) (266). PI-MECs proliferate during pregnancy and give rise to the alveolar structures (267). PI-MECs express high levels of CD49 (268), suggesting that they are a component of the population shown to repopulate the mammary gland (260).

Mouse mammary stem and progenitors cells are arranges in a hierarchical manner (Figure 3). The relationship between individual stem and progenitor cells identified by lineage tracing and cell surface markers is unclear. The number of intermediates and organization of the hierarchy is unknown.

Human Mammary Stem and Progenitor Cells

Similar to mice, the human mammary gland also contains a population of stem cells (Figure 3). Examination of X-chromosome inactivation patterns in the normal, human mammary gland suggests that entire ducts and lobules, containing both luminal

epithelial and myoepithelial components, are clonally derived from a single cell (269). Immunohistochemistry and FACS analysis of stem cell markers identifies putitive stem cells in the human mammary gland (265, 270-274). Low levels of epithelial cell adhesion molecule (Ep-CAM) and high levels of CD49f identifies a subpopulation of human mammary epithelial cells capable of generating mammary gland-like structures in severely immunocompromised mice. These experiments require “humanization” of the stromal microenvironment, achieved by pre-injection of either the cleared mammary fat pad or highly vascularized renal capsule with ECM components and irradiated fibroblasts. The resulting mammary structures contained both ductal and lobular structures composed of myoepithelial and luminal lineage cells. Serial transplantation demonstrates that these cells are capable of self-renewal (65, 275, 276). However, this system is limited by inefficiency.

Subpopulations of bipotent and lineage restricted progenitor cells have been identified in human breast tissue using *in vitro* clonogenic assays, evidencing a hierarchical organization of mammary epithelial precursors (271, 277, 278). Stem and progenitor cells are resistant to anoikis and can be propagated under non-adherent conditions, resulting in floating multicellular structures termed mammospheres. Serial passaging of mammospheres is an accepted surrogate test of self-renewal (260, 265, 270-274, 279). Disassociated mammospheres plated at clonal densities in adherent monolayer culture can give rise to colonies containing myoepithelial cells (markers include: K5, K14, and α-smooth muscle actin), luminal epithelial cells (markers include: K8, K18, K19, MUC-1, ER, and PR), or both cell types.

Using these assays the mammary precursor population isolated based on expression of EpCAM and CD49f can be further subdivided into a uncommitted/bi-potent population (Muc-1^{-}, CD24^{-}, CD133^{-}, Thy1^{+}, and CD10$^{+)}$ and luminal-restricted population (Muc-1^{+}, CD24^{+}, CD133^{+}, Thy1^{-}, and CD10^{-}) (65, 274, 275, 279-281). Bi-potent progenitor cells within the human mammary gland can also be identified by double positivity for myoepithelial K14 and luminal K19 (274). Specific markers of myoepithelial-restricted precursors have not been identified, however myoepithelial-restricted colony forming cells have been identified *in vitro* (281, 282)

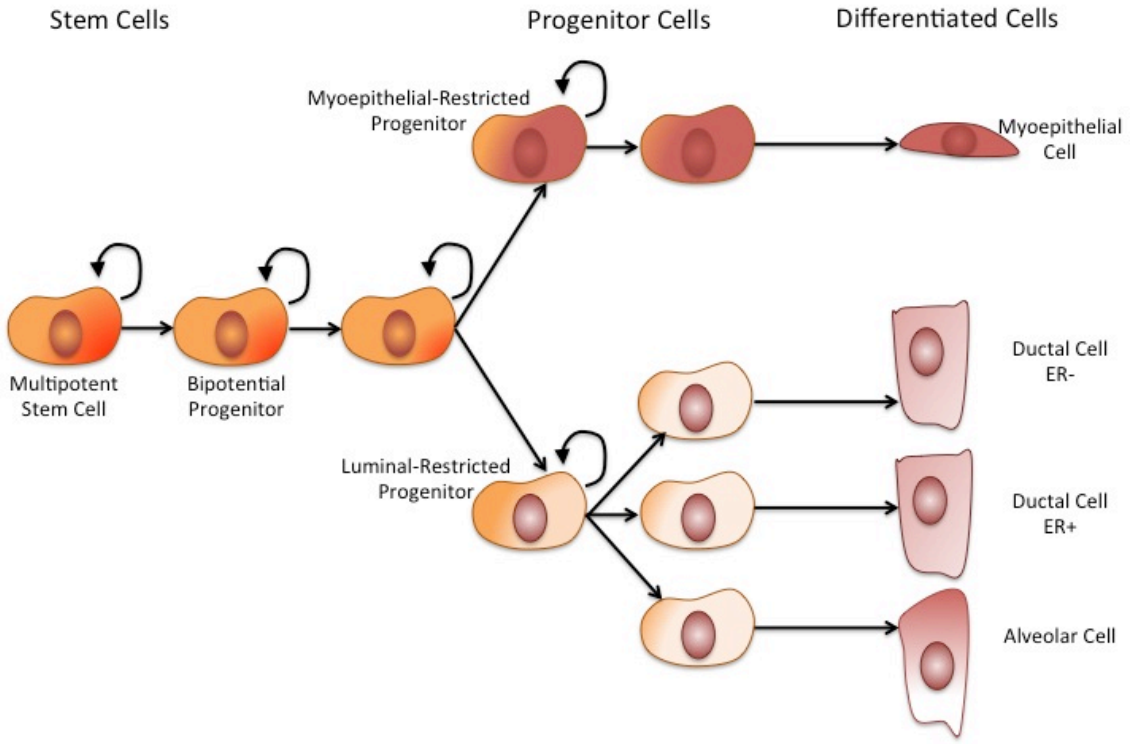

Figure 3: Simplified Mammary Stem and Progenitor Cell Hierarchy

Mouse and human stem and progenitor cells are organized in a hierarchical manner. In this simplified diagram, a multipotent stem cell gives rise to a bipotent progenitor, which in turn generates two lineage restricted progenitor populations, luminal-restricted and myoepithelial-restricted. Through a series of intermediates these progenitors give rise to the differentiated populations making up the mammary gland. The number of intermediates and the relationship between them are unknown.

Stem and Progenitor Cell Niche

In the mammary gland stem and progenitor cells exist within a specific microenvironmental niche and are not randomly distributed throughout the bulk of the epithelium. In the developing mouse mammary gland, stem and progenitor cells reside within the TEB, however in the mature mammary gland these cells are located within specialized regions of the duct as observed by immunohistochemical analysis of stem cells markers and the spatial arrangement of lobule structures during pregnancy (267, 283, 284). Microdissection of the human mammary organoids from reduction mammoplasty and validation of stem cell properties *in vitro* revealed the stem cell niche is in the terminal duct (274).

Self-renewal and the ability to differentiate into multiple cells types are advantageous traits for tumor cells. Cell extrinsic control of stem cell activity may have evolved to prevent unrestrained, cell autonomous self-renewal and carcinogenesis (285). In the absence of self-renewal signals from the niche, stem cells differentiate making it difficult to study the factors influencing mammary stem cell biology *in vitro*.

Regulation of Stem and Progenitor Cells

Although the existence of stem and progenitor cells is well accepted, the molecular mechanisms underlying their maintenance and differentiation remains unclear. Paracrine signaling downstream of hormone receptors and microenvironmental factors are critical to the maintenance and differentiation of normal mammary stem and progenitor cells.

Mammary stems cells can be enriched based on the expression of integrins, particularly α6-(CD49f) and β1-(CD29). Heterodimeric integrin complexes, such as α6/β1, directly anchor cells to the ECM and are critical to communication with the stromal microenvironment. Deletion of β1-integrin in the basal compartment (using K5-Cre) eliminates the mammary repopulating capacity in serial transplantation experiments. The mammary glands of these mice also display abnormal ductal branching, atypical polarity, and disequilibrium between the myoepithelial and luminal lineages. β1-integrin mediated interactions with the ECM are critical to maintenance and cell fate decisions in mammary stem cells (286).

Ovarian hormone exposure and reproductive history profoundly influence breast cancer risk. Although mammary stem and progenitor cells are generally ER and PR negative (only a subset, 6-10%, of luminal progenitors express hormone receptors) hormone dynamics dramatically affect the frequency and activity of these cell populations (154). Ovariectomy dramatically reduces the number of stem/progenitor cells, while treatment with estrogen and progesterone restore their numbers (287). Pregnancy enhances the stem/progenitor pool ten-fold (288). The downstream effectors of ERα and PR, amphiregulin, Wnt-4, and Rankl, are critical paracrine factors affecting mammary stem and progenitor cell dynamics (287, 289, 290) Fluctuations in the frequency and activity of stem and progenitor cells in the context of reproductive cycle and pregnancy likely underlies the complicated relationship between breast cancer risk and ovarian hormone exposure (105). The estrogen receptor target gene, Gata3, plays a specific role in the evolution of the luminal lineage. Conditional knockout of Gata3 in the mouse mammary gland results in expansion of the luminal progenitor population and defective differentiation. Overexpression of Gata3 in mammary stem cells forces differentiation into alveolar luminal cells (264). GATA3 is an important marker and is essential to the development of luminal breast cancer (291).

Transcriptional profiling of human mammary bi-potent progenitors and luminal restricted progenitors suggests that the NOTCH, WNT, and NF-κB pathways are of particular importance in the regulation of stem/progenitor cell function. The NOTCH signaling pathway is critical to stem cell maintenance and the specification of cell fate in diverse tissues. NOTCH receptor 4 was highly expressed in the bi-potent progenitor population, while NOTCH receptor 3 was enriched in the luminal progenitor populations, suggesting a role for differential NOTCH signaling in regulating the stem cell hierarchy (280). Disruption of Cbf-1/Rbpκ, a critical component of the Notch transcriptional complex, alters the cellular composition of the mouse mammary gland during pregnancy (292). Downregulation of Notch signaling in mammary stem cells results in expansion of the stem and progenitor cell pool, suggesting that Notch is required for cell fate decisions and the regulation of the stem and progenitor cell pool within the mammary gland. In progenitor cells, constitutive activation of Notch-1 results in preferential selection of luminal cell fate resulting in the accumulation of luminal progenitors, hyperplasia, and tumorigenesis (293-295). In breast cancer patients,

NOTCH1 and JAG1 overexpression have been observed and are prognostic of poor outcome (296).

The Wnt/β-catenin pathway is critical to mammary stem cell self-renewal. Wnt ligands expand the pool of mammary progenitors, however they fail to significantly alter cell fate (297). Overexpression of Wnt ligands in the mammary gland is associated with a six-fold increase in the mammary stem cell population (261). Overexpression of MMP-3 enhances the mammary stem cell population and induces hyperplasia by binding the non-canonical wnt, Wnt5b, which is known to inhibit the canonical Wnt/β-catenin pathway (298). MMP-3 knockout mice demonstrate a marked decrease in the mammary stem cell activity (298). Wnt/β-catenin is commonly deregulated in breast cancer (299-302). The hedgehog pathway also appears to regulate the stem cell population within the mammary gland; expression of constitutively activated smoothened was shown to increase the pool of stem and progenitor cells (303).

The factors governing the maintenance, proliferation, and differentiation of mammary stem and progenitor cell is poorly understood. Stem and progenitor cell populations are long-lived, compared to fully differentiated cell types. Proliferation of mammary stem and progenitor cell population in the context of mammary gland remodeling exposes them to the accumulation of transforming mutations over the lifespan of the individual. Mammary stem and progenitor cells are likely cellular targets of oncogenesis in breast cancer.

Cancer Stem Cells

According the cancer stem cell hypothesis, breast tumors are organized in a hierarchical manner, similar to normal breast tissue. In this model, only a small subpopulation of tumor cells, termed cancer stem cells, drive tumor growth, progression, recurrence, and therapeutic resistance. Cancer stem cells have been hypothesized to originate from normal tissue stem cells that have acquired a limitless proliferative capacity. Alternatively, a more differentiated cell acquires the cancer stem cell phenotype.

High levels of CD44 and low levels of CD24 enrich for breast cancer stem cells capable of recapitulating tumors in immunocompromised mice following serial transplantation at limiting dilutions (304). Gene expression analysis of cells isolated

using these markers identifies a gene signature highly enriched in stem cell regulators/markers identified in other organ systems and prognostic of poor patient outcome in breast cancer (305, 306). High activity of aldehyde dehydrogenase (ALDH) and the ability to form mammospheres in a non-adherent culture system are similarly capable of identifying cancer stem cells. Furthermore, ALDH1 expression is prognostic of poor patient outcome (307). Many genes and pathways associated with stem cell function, such as Wnt, NOTCH, NF-κB, hormone receptor signaling, are deregulated in tumors.

Breast Cancer Progression

Post-mortem histological examination of adult tissues reveals a high frequency of tumors that fail to progress to clinical relevance despite the expression of strong oncogenes (2). Occult tumors are observed in the breast tissue of approximately 40% of young and middle-aged women (20-54 years of age) (308). The high relative frequency of microscopic tumors compared to actual clinical diagnoses suggests the existence of robust tumor suppressive mechanisms limiting tumor growth and progression. Understanding the barriers to tumor growth/progression and the mechanisms utilized by tumors to circumvent them are essential to the development of novel therapeutic strategies and predictive/prognostic markers.

Oncogenes and Tumor Suppressors

Tumorigenesis is driven by genetic and epigenetic alterations. In 1982, several groups simultaneously reported the discovery of activated and transforming ras, the first oncogene (309-311). Oncogenes are normal cellular genes constitutively or inappropriately activated by chromosomal translocation, gene amplification, or mutation. In 1986, loss of heterozygosity at the retinoblastoma susceptibility locus led to the identification of the first tumor suppressor gene, Rb (312). Tumor suppressors are genes rendered insufficiently active or inactive by deletion, insertion, mutation, or epigenetic silencing. Except in rare cases where haploinsufficiency provides a selective advantage during tumorigenesis (i.e. PTEN), tumor suppressors generally require loss of both alleles (313, 314).

Multiple safeguards have evolved in mammals to impede tumorigenesis. To circumvent these safeguards, the activation of multiple oncogenes and/or loss of multiple tumor suppressors are typically required for transformation (315, 316). Tumor initiation occurs within a single cell. Stem and progenitor cell are believed to be preferential targets of transformation within the mammary epithelium.

Clonal Selection Model of Tumor Progression

Following initiation, the accumulation of additional somatic mutations is essential to tumor growth and progression. Cancer develops over many years following the initiating event, reflecting the time required for accumulation of mutations. Tumorigenesis is theorized to be an evolutionary process, driven by the selection and expansion of tumor clones with advantageous traits from a heterogeneous population. The identification of cancer stem cells suggests that only a small subset of cells are responsible for tumor growth and progression giving rise to a phenotypically diverse population of non-tumorigenic progeny cells through an aberrant differentiation program. Given that the differentiated progeny of the cancer stem cells are incapable of contributing to disease progression the clonal selection may occur exclusively among the cancer stem cells. Alternatively, plasticity between the cancer stem cell compartment and more differentiated progeny making up the bulk of the tumor may allow clonal selection and expansion across the broader tumor cell landscape to influence the disease progression.

Histopathological examination of tumor progression has defined a model in which several intermediate stages exist between initiation and a malignant tumor. In some tumor types, the acquisition of specific mutational events coincides transition between histologically defined stages. For example, colon cancer progression is characterized by the sequential accumulation of genetic alterations: APC/β-catenin mutation results in crypt dysplasia, the activation of K-RAS drives progression to an intermediate adenoma, the activation of DCC/SMAD4/SMAD2 coincides with transition to a late adenoma, and finally the loss of p53 occurs at the transition to invasive colon carcinoma (317). Breast cancer, on the other hand, demonstrates much greater heterogeneity in tumor progression and as a result very few recurrent genetic alterations have been identified in breast cancer (85, 318, 319).

Ductal Carcinoma In Situ

Strong molecular, epidemiological, and histopathological evidence suggests that DCIS is antecedent to the majority of invasive breast carcinomas. Pre-malignant atypical ductal hyperplasia (ADH) is the precursor to DCIS (320). Both ADH and DCIS are characterized by the proliferation and accumulation of neoplastic cells within the lumen of mammary ducts. ADH lesions are smaller and lack some histological features of DCIS, but are contiguous with low grade.

DCIS is a genetically advanced lesion, demonstrating a high degree of cytogenetic abnormalities (321). Many of the genetic alterations evident in invasive carcinoma are already established in pre-invasive DCIS (322-324). Comparison of DCIS and adjacent invasive carcinoma finds significant continuity between many molecular markers, including amplification of HER-2, ERα, cyclin D1, and Myc and mutations of PI3KCA and p53 (325). The intrinsic subtypes of breast cancer can be identified in pre-malignant breast tissue, suggesting the existence of distinct avenues of breast cancer progression (326). Gene expression profiling of large cohorts of DCIS and invasive breast tumors cannot resolve stage-specific differences in gene expression (318, 327-329). Based on the available evidence, specific genetic events are unlikely to facilitate the progression of DCIS to invasive carcinoma. The majority of mutational events driving tumor progression are likely acquired prior to DCIS.

Metastatic Breast Cancer

The underlying cause of death among breast cancer patients is the metastatic spread of tumors to distant organ sites, especially the bone, lungs, brain, and liver. The five-year relative survival rate of women diagnosed with localized breast cancer is 98.6 percent, decreasing to 83.3 percent for patients with regional spread, and 23.3 percent for patients with distant metastasis (31). Metastatic breast cancer can develop at any time, years or even decades after the original diagnosis and remains virtually untreatable using current therapies (330). Following surgical excision of the primary breast tumor, 20-30% of breast cancer patients will experience a metastatic recurrence over a 15-year period (331).

No reproducible genetic events can differentiate metastatic lesions from the primary tumor. Gene expression signatures predicting the development of metastatic

disease can be derived from early stage breast cancer patients (332, 333). Circulating tumor cells can be detected in a subset of these patients. Metastases can be generated from these genetically 'primitive' clones of the primary tumor (334-336). These results indicate that metastatic propensity is likely programed early during breast tumorigenesis, by many of the same genetic and epigenetic events that drive proliferation and clonal expansion (337). Multistep progression models predict that alteration in metastasis genes late in tumorigenesis triggers dissemination. These models provide the rationale for mammographic screening and early intervention in breast cancer. However, the available data suggests that the manifestation of breast cancer as a metastatic lesion is unlikely to be a result of additional mutational events late in tumorigenesis (332-336).

Role of the Microenvironment in Breast Cancer Progression

Accumulating evidence suggests that the normal microenvironment has tumor suppressive properties. Injection of fully malignant mammary carcinoma cells into the embryonic mammary mesenchyme can suppress tumor formation and induce differentiation (6). Malignant teratocarcinoma cells injected into a normal developing mouse blastocyst give rise to phenotypically normal chimeric mice (5). Similarly, chicken embryos infected with Rous Sarcoma Virus (RSV) develop normally despite constitutive expression of the v-src oncogene. Dissociation of the embryo alleviates restraints on transformed growth (4). Tumor formation in RSV-infected chickens requires wounding, suggesting a role for an altered microenvironment in tumor progression (3). Even genotoxic agents target the normal microenvironment in animal models of carcinogenesis. The cleared mammary fat pad of carcinogen-exposed rats enhances the tumorigenicity of transplanted mammary epithelial cells exposed to carcinogens *in vitro.* By comparison, the mammary fat pad of un-exposed rats suppressed the tumorigenic potential of identically *in vitro* transformed mammary epithelial cells instead resulting in the formation of a normal mammary ductal tree (338).

The focus on mutational events within the tumor epithelium as the primary drivers of tumor progression has largely obscured the importance of the stromal microenvironment. The ability of tumor cells to recruit and manipulate non-malignant cell types, including fibroblasts, endothelial cells, pericytes, adipocytes, mesenchymal

cells, and leukocytes, governs their malignant growth potential. In mouse models, injection of tumor cells into different anatomical sites, with varying stromal compositions, dramatically affects proliferation, invasion, metastasis, and therapeutic sensitivity (339-343). Depletion of specific non-malignant cell types or contributed factors can profoundly effect tumor progression (344-347). Many microenvironmental changes are permanent, breast tumor-derived stromal fibroblast maintain their abnormal phenotype in culture (348). Epigenetic reprogramming of stromal cell types has been shown to account for the alterations in gene expression (349). Genetic alterations have been also noted within the stromal compartment (350-352).

Critical modes of communication between tumor cells and the stromal microenvironment include, the composition of the ECM, cell-to-cell contact, and paracrine signaling molecules. Comparison of distinct cell types within the normal breast, DCIS, and invasive breast cancer, reveals significant changes in stromal-epithelial crosstalk during breast tumor progression (353). Gene expression analysis of the breast tumor stroma identifies a robust prognostic signature, evidencing the importance of stromal signaling especially in metastatic proclivity (7). In breast cancer xenograft models, the gene signature of cells migrating to the bone, lung, or brain is heavily populated by secreted or cell surface proteins, including cytokines/chemokines, MMPs, and cell adhesion molecules (354-356).

Changes in the interaction between tumor cells and the microenvironment are critical to the transition from pre-invasive DCIS to invasive breast cancer. The disappearance of myoepithelial cells as a continuous entity surrounding the duct is the critical histological distinctions of invasive disease (357, 358). Abnormalities in myoepithelial cells results in the expression of MMPs resulting in the degradation of the basement membrane (353, 357).

Deregulated interaction between the tumor epithelium and the tumor stromal are essential to tumor progression. Breast cancer progression cannot be defined by solely focusing on genetic changes within the tumor epithelium. The identification of microenvironmental factors that promote breast cancer progression is essential to the design of novel therapeutic strategies and continued progress against breast cancer mortality.

Inflammation and Breast Cancer Progression

Inflammation and the presence of leukocytes in the tumor microenvironment are regarded as essential components of malignant progression. Tumors resemble “wounds that do not heal” (359). In many tumor types, chronic inflammatory stress due to viral or bacterial infection, autoimmune disease, or environmental irritants promotes the development and progression of tumors. Helicobacter pylori infection dramatically increases risk of developing gastric cancer (360). Autoimmune inflammatory bowel disease greatly enhances colon cancer development (361). Prostatitis increases the risk of developing prostate cancer (362). Asbestos and silica particles induce chronic inflammation essential to the development of mesothelioma (363). Long-term usage of non-steroidal anti-inflammatory drugs decreases the overall burden of cancer (364-366). Systemic markers of inflammation are prognostic of poor overall and disease-free survival in breast cancer patients (367).

Many factors contribute to the inflammatory tumor microenvironment. Tumor cells secrete cytokines, chemokine, and toll-like receptor ligands downstream of oncogenes, such as RAS and MYC (368-371) and in response to stress conditions, including hypoxia, necrosis, chemotherapy, and ionizing radiation (372-374). Tumor associated fibroblast significantly contribute to the inflammatory microenvironment. Gene expression characteristic of activated fibroblasts defines a wound healing signature that is prognostic of poor survival and the development of metastasis (375). Co-injection of tumor cells with activated fibroblast is routinely used to enhance the establishment of xenograft tumors (376).

The inflammatory mileu surrounding the developing tumor results in the recruitment of leukocytes. Both innate and adaptive immune cells are evident in the tumor stroma including macrophages, dendritic cells, neutrophils, myeloid derived suppressor cells, mast cells, natural killer cells, T-lymphocytes, and B-Lymphocytes. Leukocytes can participate in either a tumor-promoting inflammatory response (demonstrated for all except natural killer cells (377)) or an anti-tumor immune response. The pro/anti-tumor properties of leukocytes are largely programmed by secreted factors and heterotypic interactions within the tumor microenvironment. In malignant tumors the balance is largely tilted in favor of a tumor-promoting inflammatory response. Many mechanisms play a role in immunosuppression within tumors including

the cytokine, TGF-β1 (378). Targeting immunosuppressive receptors expressed by T-cells, PD-1 and CTLA-4, is a promising therapeutic strategy and highlights important efforts to reactivate anti-tumor immunity (379-382).

Tumor-associated macrophages (TAMs) are among the most abundant immune cells within the tumor microenvironment. TAMs are essential to angiogenesis, invasion, and metastasis contributing cytokines, proteases, and growth factors to the tumor microenvironment. Within breast tumors, TAMs frequently localize near the vasculature. In this context, TAMs facilitate angiogenenic reprograming through secretion of VEGF and invasion by establishing chemotactic gradients of EGF (383-386). Gene expression signatures associated with poor patient outcome often include genes that are specifically expressed by macrophages (387). High levels of TAMs are associated with poor patient prognosis in breast and other tumor types (388-392). The expression of cytokines and chemokines, such as CCL2 and CSF-1, are essential to the recruit and activation of macrophages, correlated with poor patient prognosis (393-395). Depletion of macrophages in transgenic and xenograft models of breast tumorigenesis demonstrate reduced growth, invasion, angiogenesis, and metastasis (344, 396-399).

Macrophages are differentiated in multiple phenotypes M1 macrophages activated by IFNγ and microbial components are pro-inflammatory, express major histocompatibility complex molecules and likely function in anti-tumor immunity. The majority of TAMs are believed to be of the M2-phenotype, alternatively activated by IL-4, IL-10, and IL-13. M2-macrophages normally participate in the wound healing through the secretion of growth factors and angiogenic factors. In some models, tumor progression is dependent on a switch from the M1 to M2 phenotype (400). TAMs express high levels of pro-inflammatory cytokines reminiscent of the M1 phenotype suggesting that they do not fall into typical classifications. TGFβ is a critical factor in suppressing the role of TAMs in anti-tumor immunity and in the generation of a tumor promoting phenotype (401-403). Subsets of T-lymphocytes known to promote breast cancer in animal models and correlated with poor prognosis in breast cancer, progression enhance metastatic progression by stimulating the activity of TAMs through production of IL-4 and (404). TAMs stimulated by CSF-1 induces the expression of hypoxia-associated iNOS and arginase-1 which suppress the proliferation and activation of cytotoxic T-cells (405, 406).

Tumor-associated neutrophils (TAN) are also recruited by tumor cell-derived chemokines, especially IL-8 (368, 407). High levels of circulating neutrophils, induced by tumor-derived GM-CSF and other cytokines, are prognostic of poor survival in melanoma and renal carcinoma patients (408, 409). Relatively few studies have examined the prognostic significance of TAN. In renal cell carcinoma, TAN are prognostic of short recurrence-free survival (RFS). In the presence of TAN, patients demonstrated a five-year RFS of 53%, however in the absence of TAN patients had a five-year RFS of 87%. In multivariate analysis, TAN were shown to be an independent prognostic marker of disease-specific survival (DSS) and overall survival (OS) (410). In human gliomas, high numbers of TAN correlate with high tumor grade (411). High levels of neutrophils in the bronchoalveolar lavage fluid from patients with bronchioloalveolar carcinoma were associated with high levels of IL-8, NE, and poor DSS (407). Although not commonly observed in pancreatic tumors, TAN were shown to be associated with the relatively aggressive micropapillary and undifferentiated subtypes (412). Lymphocyte to neutrophil ratio is a prognostic factor in colorectal cancer and non-small cell lung cancer (413, 414).

Depletion of neutrophils or disruption of neutrophil chemotaxis inhibits tumor growth, angiogenesis, and metastasis in mouse models of tumor progression (415-417) The majority of studies in humans and animal models show that TAN augment tumor progression through the secretion of growth factors, cytokines, ROS, and proteases (345, 418-421). TAN can be polarized by TGF-β blockade to assume an anti-tumor phenotype, however little evidence exists of a role for TAN in anti-tumor immunity in the absence of therapeutic manipulation (422, 423).

Breast Cancer Subtypes

Breast cancer is a collection of diseases sharing a common anatomical site but radical differences in incidence, morphology, genetics, therapeutic response, and outcome. Clinically, breast tumors are generally segregated into three groups, estrogen receptor (ER)-positive, HER2-amplified, and triple-negative breast cancer (TNBC: ER-, PR-, and HER2-negative); these distinctions are largely based on outcome and response to available targeted therapeutics. Gene expression profiling, using DNA microarray or RNA sequencing technologies, has the capacity to rapidly measure global

mRNA transcription. Comparative study of large cohorts of tumors by gene expression profiling has greatly enhanced our understanding of breast cancer biology and heterogeneity. These studies consistently highlight the importance of differences in hormone receptors, HER2, and proliferation in breast cancer classification (332, 333, 424-426)

Pioneering work by Perou and colleagues was among the first to use these high throughput technologies to classify breast tumors and remains the most influential. Their analysis first compared gene expression between breast tumors to define an intrinsic gene set, genes demonstrating the greatest differences in expression across the tumor spectrum. Using this intrinsic gene set the tumors were then subjected to hierarchical clustering, grouping tumors by similarities in gene expression. From this type of analysis five, reproducible intrinsic subtypes of breast cancer are widely considered: luminal A, luminal B, basal, HER2-enriched, and normal-like (291, 427). Recent publications also recognize a claudin-low subtype of breast cancer (428). Analysis of breast tumors using DNA copy number array, DNA sequencing technologies, global DNA methylation analysis, microRNA sequencing, and proteomic analysis can reproducibly group tumors into these subtypes (85). Each molecular subtype displays dramatic differences in incidence, patient outcome, and characteristic gene expression (Table 1) (425, 429-431). The intrinsic subtypes of breast cancer can be identified in DCIS evidencing distinct pathways of tumor progression (319).

Molecular Subtype	Frequency	Prognosis	Survival (12 years)	Characteristic Genes	Typical Biomarker Profile
Luminal A	50-60%	Good	80%	ESR1, GATA3, KRT8, KRT18, XBP1, FOXA1, TFF3, CCND1, LIV1	ER^{+},PR^{+},$HER2^{-}$, $Ki67^{low}$, $CK8/18^{+}$
Luminal B	10-20%	Intermediate	50%	ESR1, GATA3, KRT8, KRT18, XBP1, FOXA1, TFF3, SQLE, LAPTM4B	ER^{+},PR^{+},$HER2^{-/+}$, $Ki67^{high}$, $CK8/18^{+}$
HER2-enriched	10-20%	Poor	60%	ERBB2, GRB7	ER^{-},PR^{-},$HER2^{+}$, $Ki67^{high}$
Basal-like	10-20%	Poor	35%	KRT5, CDH3, ID4, FABP7, KRT17, TRIM29, LAMC2, EGFR, KIT	ER^{-},PR^{-},$HER2^{-}$, $Ki67^{high}$,$CK5/6^{+}$, $EGFR^{+}$, $c\text{-}Kit^{+}$
Claudin-low	<10%	Poor	25%	CD44, SNAI3	ER^{-},PR^{-},$HER2^{-}$, $Ki67^{low}$,Claudin $3,4,7^{-}$, $ALDH^{+}$, $CD44^{high}$, $CD24^{low}$
Normal-like	<10%	Intermediate	ND	PTN, CD36, FABP4, AQP7, ITGA7	ND

Table 2: The Intrinsic Subtypes of Breast Cancer.

Luminal Subtypes of Breast Cancer

In general, the luminal subtypes are defined by expression of ER-complex components and target genes, including ESR1, GATA3, FOXA1, XBP1, and MYB. The luminal subtypes also highly express transcripts characteristics of the luminal lineage within the normal breast, such as cytokeratins 8/18. Luminal-subtype tumors are predominately ER-positive by immunohistochemistry (Lum A: 87% and Lum B 82%), however can contain TNBC (Lum A: 7% and Lum B 1%) and HER2-positive patients (Lum A: 2% and Lum B 15%). Segregation into luminal A and B is largely based on the differential expression of proliferation genes, which tend to be highly expressed in luminal B tumors relative to luminal A tumors. In ER-positive tumors, markers of proliferation (i.e. Ki67) can distinguish between Luminal A and Luminal B tumors (432, 433). Luminal B tumors also tend to express lower relative levels of ER-target genes. A subset of luminal B tumors demonstrates HER2 amplification.

The luminal A subtype is the largest tumor cohort (40-60% of the total) and demonstrates the greatest heterogeneity in gene expression. Luminal A tumors are characterized by low histological grade and have a relatively good prognosis. Luminal B subtype tumors are generally of higher histological grade and have an intermediate prognosis compared to all other subtypes. Luminal A patients have a 15-year relapse rate of 27.8% compared to 42.9% for luminal B patients. Following relapse, luminal A patients had a longer median survival of 2.2 years compared to 1.6 years for luminal B patients (425, 429-431).

HER2-Enriched Subtype of Breast Cancer

The HER2-enriched subtype generally demonstrates a high degree of 17q12 amplification (71%) resulting in the overexpression of HER2 and adjacent genes. The majority of HER2-enriched subtype tumors, when evaluated by immunohistochemistry are HER2-positive and ER-negative (68%), but can contain ER-positive (20%) and TNBC tumors (9%). HER2 amplicon associated gene expression is an important component of the segregation of this subtype by transcriptomic analysis. Tumors in the HER-2 enriched subtype display overexpression of receptor tyrosine kinases, including FGFR4 and EGFR, and do not express luminal cluster genes (i.e. GATA3 and ESR1). The HER2-enriched subtype is largely defined by tumors of high histological grade, high

proliferation index, and is frequently metastatic. Historically, HER-2 enriched tumors have a poor prognosis (52.4% relapse, 0.7 year median survival following relapse), however HER-2 directed therapies have increased survivorship in both metastatic and non-metastatic tumors.

Basal-like Subtype of Breast Cancer

Basal-like tumors generally lack gene expression associated with the ER, PR, and HER2 (80%), however can contain ER-positive (10%) and HER2-positive tumors (1%). Basal-like tumors express markers of the basal/myoepithelial cell lineage in the normal breast, including cytokeratins 5/17, P-cadherin, caveolin 1, nestin, CD44, and EGFR. Evaluation of basal cytokeratins and EGFR can refine the ability to identify basal-like tumors by IHC (434). Basal-like tumors are often large at diagnosis, have a high histological grade, high proliferation index, and a high frequency of lymph node involvement (425, 429-431). Basal-like tumors have a poor prognosis, 43.1% relapse with a 0.5 year median survival following relapse. A significant portion of hereditary BRCA1 and BRCA2 related tumors cluster within the basal subtype. Several studies have identified BRCA1 and BRCA2 downregulation, through either promoter methylation or transcriptional inactivation, in sporadic cases of basal-like breast cancer (61, 62). Conditional knockout of Brca1 and Trp53 in the mouse mammary gland generates tumors with distinctly basal-like features (63-65).

Additional Subtypes of Breast Cancer

Normal-like tumors cluster with normal breast tissue, have not been extensively characterized. The normal-like subtype may be an artifact of normal tissue contamination and is generally ignored (431).

Since the original description of the intrinsic subtypes of breast cancer, several additional subtypes have been described, including the interferon subtype characterized by high levels of interferon gene expression and the molecular apocrine subtype characterized by androgen receptor signaling. Notable is the claudin-low subtype. These tumors cluster closely with the basal-like tumors, however they characteristically express very low levels of intracellular adhesion molecules including claudin-3,4,7, ocludin, and e-cadherin. These tumors are of high histological grade and low

proliferation index. Claudin-low tumors are enriched for mesenchymal and cancer stem cell properties and highly express genes characteristic of immune cell infiltration (428). Claudin low patients have a very poor prognosis.

A recent study identified six-subtypes of TNBC through k-means and consensus clustering of gene expression profiles. Two basal-like tumor subtypes, basal-like 1, basal-like 2, were characterized by high expression of genes associated with cell proliferation and the DNA damage response making them extremely responsive to chemotherapy. An immunomodulatory subtype was identified by the presence of immune cell signaling. A mesenchymal and mesenchymal stem-like were defined by cell signaling characteristic of epithelial to mesenchymal transition and cancer stem cells. Finally a luminal androgen receptor (AR) subtype was identified expressing luminal cytokeratins, but lacking ER-signaling in favor of an AR gene signature (435).

Although the intrinsic subtypes of breast cancer are prominently considered, a great deal of heterogeneity exists beyond the luminal A, luminal B, HER-2 enriched, and basal-like distinction. The ability to classify tumor heterogeneity through molecular profiling may allow cancer oncologists to identify effective therapeutic approaches to similar tumors. The analysis of breast tumors by multiple profiling platforms and using new bioinformatics techniques should continue to reveal sub-classifications of breast cancer, predict outcomes, and guide clinical management.

Recurrent Genetic Abnormalities Associated with Breast Cancer Subtypes

Breast tumors are genetically heterogeneous, however some recurrent abnormalities cluster within the intrinsic subtypes of breast cancer. Luminal A tumors (Figure 4) demonstrate the lowest overall mutation rate and generally have diploid genomes. Luminal B tumors (Figure 5) have an intermediate mutational rate and aneuploidy genomes. Recurrent mutations in the luminal subgroups include: PIK3CA (Lum A: 49%, Lum B: 32%), GATA3 (Lum A: 14%, Lum B: 15%), MAP3K1 (Lum A: 13%, Lum B: 5%), and MAP2K4 (Lum A: 7%, Lum B: 2%). Mutations in p53 are more common in luminal B tumors (Lum A: 12%, Lum B:32%), overall the majority of luminal B tumors have some kind of inactivating events within the p53 pathway. Accordingly, luminal A tumors express higher levels of p53 target genes GADD45 and CDKN1A. The Rb-pathway inactivation is also higher in luminal B tumors. Common oncogenic events

in luminal B tumors include cyclin D1 (58%) and CDK4 (25%) amplification (compared to luminal A tumors cyclin D1 amp: 29% and CDK4 amp. 14%). Inactivating mutations in RB1 are also more common in luminal B tumors. Overall, differences in the clinical behavior of luminal A and B tumors can partially be explained by differences in Rb and p53 inactivation.

HER2-enriched (Figure 6) and Basal-like (Figure 7) tumors demonstrate significantly higher mutational rates compared to luminal tumors and often have aneuploid genomes. Overall, TP53 mutations are highly enriched in basal-like and HER2 tumors (Basal:84%, HER2:75%). RB1 mutation/loss is a frequent event in basal-like tumors (20%) as is cyclin E amplification (9%); interestingly cyclin D1 amplification is rare in the basal like subtype compared to luminal tumors and HER2 enriched tumors (cyclin D1 amp. 38%, CDK4 amp. 24%). PIK3CA mutations are less frequent in basal-like tumors compared to luminal and HER2 enriched subtypes (Basal: 7%, HER2: 42%). However, basal like tumors display the highest Akt activity due in part to frequent PTEN/INPP4B loss and amplification of PIK3CA (49%). Basal-like tumors also display amplification of genes within the ERK signaling pathway, including KRAS, BRAF, and EGFR.

The spectrum of genetic alterations plays an important role in breast cancer heterogeneity. However, alterations are often shared between subtypes. Unique mutational spectra does not account for the vast differences in gene expression that drive the segregation of tumors into the intrinsic subtypes.

Cell of Origin and Breast Cancer Subtypes

Differences in gene expression that segregate the distinct subtypes of breast cancer are believed to arise from different cells of origin. Analysis of histological and molecular profiles of breast tumors does not necessarily predict the normal cell of origin. BRCA1 mutation carriers are particularly susceptible to basal-like breast cancer; therefore the assumption was that BRCA1 mutation leads to the transformation of basal progenitor cells. Inactivation of BRCA1 and p53 in basal and luminal progenitor cells demonstrates that luminal progenitors are the target of BRCA1 loss and the genesis of basal like breast cancer (65). The cell of origin responsible for the genesis of the other breast cancer subtypes have not been convincingly demonstrated.

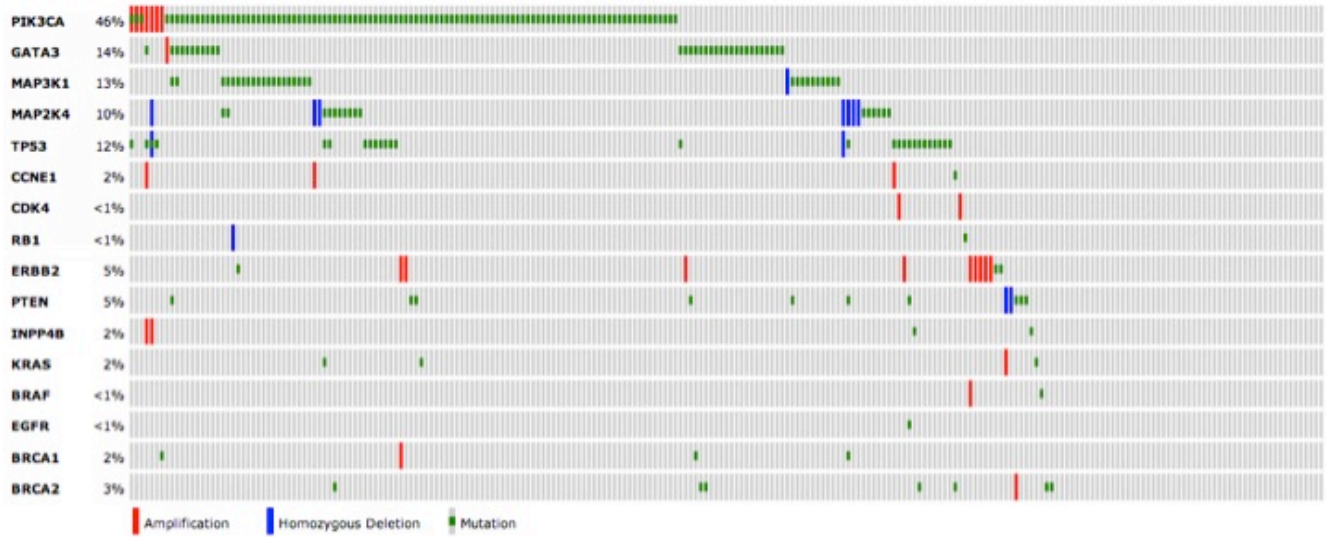

Figure 4: Common Genetic Alterations in Luminal A Breast Tumors.

Amplifications, deletions, and mutations in recurrently altered genes within the luminal A subtype of breast cancer. Alterations were measure by exome sequencing and array comparative genomic hybridization (CGH) by TCGA. The data was visualized as presented here using the web-based cBioPortal for cancer genomics (www.cbioportal.org).

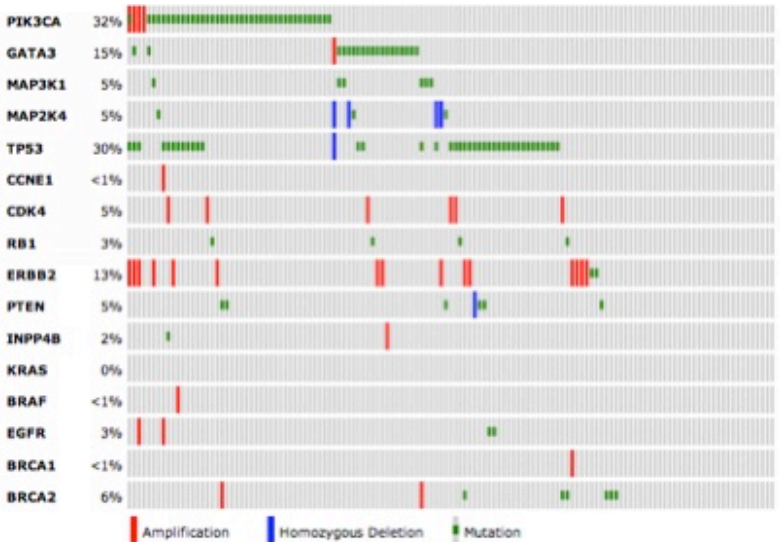

Figure 5: Common Genetic Alterations in Luminal B Breast Tumors.

Amplifications, deletions, and mutations in recurrently altered genes within the luminal B subtype of breast cancer. Alterations were measure by exome sequencing and array comparative genomic hybridization (CGH) by TCGA. The data was visualized as presented here using the web-based cBioPortal for cancer genomics (www.cbioportal.org).

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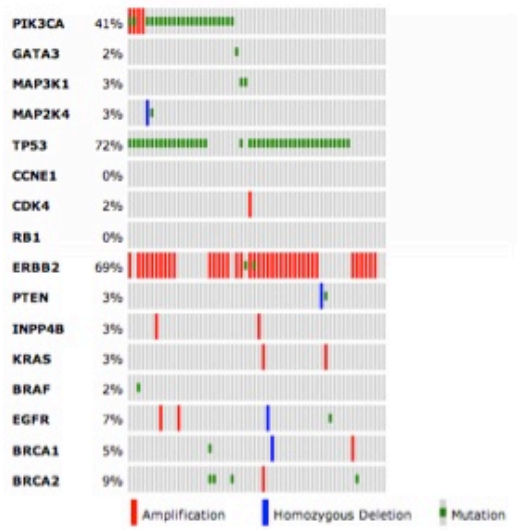

Figure 6: Common Genetic Alterations in HER2-Enriched Breast Tumors.

Amplifications, deletions, and mutations in recurrently altered genes within the HER2-enriched subtype of breast cancer. Alterations were measure by exome sequencing and array comparative genomic hybridization (CGH) by TCGA. The data was visualized as presented here using the web-based cBioPortal for cancer genomics (www.cbioportal.org).
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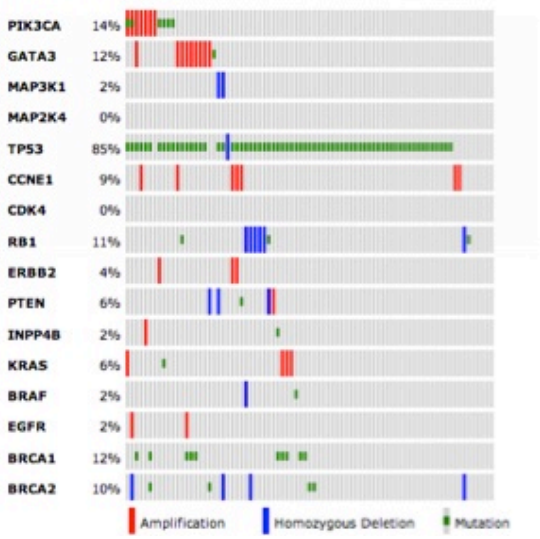

Figure 7: Common Genetic Alterations in Basal-Like Breast Tumors .

Amplifications, deletions, and mutations in recurrently altered genes within the basal-like subtype of breast cancer. Alterations were measure by exome sequencing and array comparative genomic hybridization (CGH) by TCGA. The data was visualized as presented here using the web-based cBioPortal for cancer genomics (www.cbioportal.org).
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Clinical Management of Breast Cancer

Breast cancer remains a serious public health concern. In the United States, one in eight women will be diagnosed and treated for breast cancer within her lifetime. Declining mortality rates over the last several decades reflects progression in the clinical management of the disease (31). Prior to the mid-1970s breast cancer treatment had seen little change since the introduction of the radical mastectomy by William Halsted in 1882. Over the last several decades increased breast cancer survivorship has come from advances in screening/diagnostic imaging, surgical resection, evaluation of prognostic/predictive markers, radiotherapy, systemic chemotherapy, and targeted therapeutics.

Mammography

Mammographic screening has contributed to the decline in breast cancer mortality, however the effect is often overstated. Overall, the number of women who present with advance breast cancer has decreased only eight-percent due to screening mammography (436). The success of early detection is predicated on the linear progression of breast cancer from initiation to metastatic disease. However, metastatic proclivity may be pre-programed early during tumorigenesis, resulting in early dissemination of tumor cells. Mammography overwhelmingly detects slow-growing primary tumors, a significant proportion of which are unlikely to progress within the lifespan of an individual (437). The molecular mechanisms governing the tumor proliferation rate and metastasis are linked, therefore the tumors with the greatest proliferation rate may also be the most likely to metastasize and least likely to be detected by mammography (438, 439). Increased sensitivity and access to mammographic screening has contemporaneously led to the detection of a larger proportion of pre-invasive DCIS (440). DCIS currently accounts for 20% of all breast cancer diagnoses (440). Examined retrospectively, breast cancer screening results in over-diagnosis in 31% of patients. Prior to the widespread use of mammography DCIS was a very rare diagnosis (436). The development of complimentary screening methodologies is necessary to accurately define patient risk of disease progression and guide therapeutic intervention.

Breast Cancer Diagnosis and Assessment of Clinicopathological Parameters

Diagnostic imaging and core needle biopsy are performed to determine the presence of malignancy. Following diagnosis, breast cancer treatment is based on stage and histopathological parameters. Tumor stage, the most important prognostic parameter, is based on evaluation of tumor size, the number/proximity of involved lymph nodes, and the presence or absence of distant metastasis. Guidelines for the evaluation of tumor stage are set by the American Joint Committee on Cancer (AJCC). Tumor stage is significantly associated with patient outcome and is a critical tool utilized by oncologists to make treatment decisions (Table 2).

In the absence of metastasis pathological features of the tumor, including histological grade, hormone receptor (ER and PR), HER2 status, and the presence of lymphovascular invasion are heavily considered in making treatment decisions. Platforms including the PAM-50 Breast Cancer intrinsic classifier, the MammaPrint test, and the Oncotype DX are now being used to supplement traditional clinicopathological assessment. However, the adoption of these diagnostic platforms is limited by their substantial cost, lack of standardization, and incomplete clinical trials comparing outcomes of patients treated according to the established risk groups.

Stage	Five-year Overall Survival (441)	Description
0	93%	Carcinoma *in situ*
I	88%	1A: The tumor is less than 20 mm in the greatest dimension with no evidence of axillary lymph node metastasis. 1B: The tumor is greater than 20 mm with micrometastasis (<2mm) to a single axillary node.
IIA	81%	The tumor is either between 20 and 50 mm or less than 20 mm with 1-3 positive lymph nodes.
IIB	74%	The tumor is either greater than 50mm or between 20 and 50mm with 1-3 positive node.
IIIA	67%	The tumor is either less than 50 mm and has 4-9 positive nodes or a tumor is greater than 50 mm and has 1-3 positive nodes,
IIIB	41%	The tumor extends to the chest wall or the skin.
IIIC	49%	A tumor of any size with more than 10 positive nodes.
IV	15%	Stage IV infers distant metastasis.

Table 3: Breast Cancer Staging and Survival.

Tumor stage is the most important prognostic parameter available to clinicians. Tumor stage is based on the size of the tumor, the frequency of lymph node metastasis, and the presence of distant metastasis.

Surgery

Breast conserving surgery (BCS), lumpectomy followed by radiation therapy is the gold standard. Large, randomized clinical trials have consistently failed to find a difference in local reoccurrence rates and overall survival between BCS and modified radical mastectomy (442-446). The morbidity associated with axillary lymph node dissection can also be avoided if biopsy of the sentinel lymph node, the node directly receiving drainage from the tumor, is negative for metastasis. Randomized clinical trials demonstrate no difference in the outcome of patients who undergo sentinel lymph node biopsy to determine if complete axillary lymph node dissection was necessary compared to complete dissection regardless of sentinel lymph node positivity (447, 448). These studies have been practice changing such that complete node dissection will now be performed only in those patients with sentinel lymph node involvement.

Radiotherapy

Radiotherapy, a necessary component of breast conserving surgery, is administered to the entire breast to prevent local recurrence. Typically, external-beam radiation therapy is used to apply a total dose of 50 Gy to the breast over a five-week period in 2-Gy fractions. Radiation therapy reduces five-year local recurrence from 26% to 7% in the conserved breast and decreases 15-year breast cancer mortality to 30.9% compared to 35.9% for women who did not receive radiation therapy (442). An additional dose of 16 Gy directed at the tumor bed reduces the risk of recurrence even further from 7.3% to 4.3% at five years (449).

Chemotherapy

Systemic chemotherapy is frequently used to palliate the symptoms of patients with advanced breast cancer and to reduce the risk of recurrence in patients with operable breast cancer. The median survival of metastatic breast cancer patients is 18-24 months. Treatment with systemic chemotherapy achieve complete responses in 16.6% of patients of which only 3.1% will remain in remission after 5-years (450). Chemotherapeutics are generally administered in combination to increase therapeutic efficacy. In randomized clinical trials, many combinations have been tested for efficacy

and tolerability. In advance breast cancer patients, these studies generally observe greater response rates with combination therapy compared to mono-therapy, however with increase toxicity.

For breast cancer patients the risk of recurrence spans decades following diagnosis and treatment. Following breast conserving surgery, 10-20% of patients will experience recurrence of those 9-25% are metastatic or locally advance (451-453). Adjuvant chemotherapy, administered after surgical resection, reduces the risk of developing distant metastasis by eliminating pockets of tumor cells within the body. However, the clinicopathological parameters used to determine risk are incomplete and fail to adequately stratify individual risk resulting in the indiscriminate application of adjuvant chemotherapy. As many as 80% of treated patients would never actually form metastases and needlessly suffer the morbidity associated with systemic chemotherapy (454).

Neoadjuvant chemotherapy, administered prior to surgical resection, was originally used in patients with locally advanced disease to enhance operability, but is now more widely used in the treatment of operable breast tumors. A pathological complete response is achieved in 20-30% of patients treated with neoadjuvant chemotherapy. The response of breast tumors to chemotherapy is highly variable. A pathological complete response is achieved in 28-32% of TNBC and HER-2-enriched subtype patients, partially due to their high proliferation rate. In contrast, luminal tumors respond poorly to neoadjuvant chemotherapy, a pathological complete response is achieved in only 6.7% of luminal A patients and 11.2% of luminal B (HER2-negative) (455). Neoadjuvant therapy has the added advantage of allowing oncologists to evaluate the efficacy of the drugs being administered by diagnostic imaging, allowing them to modify the dose or the drugs being applied if ineffective.

The earliest adjuvant chemotherapy trials utilized a combination of cyclophosphamide, methotrexate, and 5-flourouracil (CMF) to successfully increase disease specific and overall survival compared to surgery alone in a cohort of lymph node positive breast cancer patients (456). CMF was eventually supplanted by combination regimens containing anthracyclines, especially doxorubicin and epirubicin. In a randomized clinical trial, anthracycline regimens demonstrated an 11% reduction in the risk of recurrence compared to CMF, however others studies are inconclusive on

the benefits of anthracyclines in combination therapy (331, 457, 458). Overall meta-analysis of clinical trial using both the CMF and anthracycline based adjuvant chemotherapy regimes demonstrates a reduction in recurrence rate of 35% in women younger than 50 and 20% in women older that 50. Adjuvant chemotherapy increases 10-year survival 7% (71% to 78%) in node negative patients and 11% (42% to 53%) in node positive patients (458). The addition of taxanes, docetaxel and paclitaxel, has further boosted the efficacy of anthracycline based adjuvant chemotherapy, increasing disease free survival 14% over five years (459).

Large randomized trials testing adjuvant versus neoadjuvant chemotherapy fail to find a significant difference in disease-free or overall survival compared to adjuvant chemotherapy. However, neoadjuvant therapy does increase the percentage (7-12%) of patient eligible for breast conserving surgery without an increase in locoregional recurrence (460-462). Neoadjuvant therapy has had an important impact on translational research and clinical trial design, allowing novel therapies and therapeutic combinations to be tested on a smaller number of patients and with shorter follow-up compared to traditional adjuvant trails. Neoadjuvant trials are evaluated based on tumor response and permit collection of tumor tissue before/during treatment by serial biopsy and after surgical resection in patients not undergoing a pathological complete response. These trials are important to the development of predictive markers and optimization of therapy.

Targeted Therapeutics

Traditional chemotherapy is non-specific, targeting proliferating populations of tumor and normal cells, and is therefore associated with significant toxic side effects. Breast cancer was among the first tumor types to benefit from targeted therapeutics. The link between breast cancer and the endocrine system was established in 1896 when it was observed that ovariectomy induced tumor regression in a subset of breast cancer patients. In 1952, adrenalectomy was found to produce a similar response in breast tumors. These early studies failed to identify the biological basis of these observations. Identification of the role of the estrogen in breast cancer and the introduction of therapeutics specifically inhibiting ER signaling has significantly

enhanced survivorship in the subsets of breast cancer patients expressing ER (331, 437, 463-467).

Selective estrogen receptor modulators (SERMs), such as tamoxifen, structurally resemble estrogen; however upon binding to the ER inhibit its ligand-dependent functions. In metastatic breast cancer patients (pre-menopausal) the efficacy of tamoxifen is identical to bilateral ovariectomy (468). The tamoxifen response rate of ER-positive metastatic breast cancer is 30%, for both pre- and post-menopausal women (469). Long-term, administration of tamoxifen to ER-positive patients as adjuvant therapy cuts the recurrence rate in half and reduces mortality by a third over a 15-year period (331). Treatment of DCIS patients with tamoxifen significantly reduces disease progression and prevents breast cancer in a cohort of high-risk patients (466, 470). In some tissues tamoxifen acts as a partial agonist of ER, increasing the risk of developing endometrial cancer or a thromboembolic event (471, 472).

Aromatase inhibitors, including anastrozole, letrozole, and exemestane, have been developed as alternatives to tamoxifen. Aromatase inhibitors interfere with the enzymatic conversion of androgens to estrogen and are therefore only effective in the treatment of post-menopausal women. In ER-positive metastatic breast cancer, aromatase inhibitors prolonged survival in women who fail to respond to tamoxifen (473-476). As first line therapy for metastatic breast cancer, aromatase inhibitors demonstrate greater clinical efficacy than tamoxifen alone (477, 478). In a large randomized, double-blinded study, anastrozole was compared to tamoxifen as adjuvant therapy in post-menopausal women. Anastrozole demonstrated greater clinical efficacy in the prevention of breast cancer recurrence (479-481). Fulvestrant, an antagonist that results in the downregulation of the ER and the PR, is another alternative to tamoxifen in the treatment of breast cancer. Fulvestrant demonstrates equivalent efficacy to anastrozole in patients resistant to tamoxifen (482)

The receptor tyrosine receptor HER2 is amplified in approximately 30% of breast tumors. HER2 amplification is associated with aggressive disease and a poor survival. Introduction of the HER2-directed chimeric monoclonal antibody, trastuzumab, has dramatically improved the outcome of women with HER2-amplified breast tumors (both ER+ and ER-). Trastuzumab decreases the recurrence rate by 50% and reducing

mortality by a third (464). The dual (HER2/EGFR) kinase inhibitor, lapatinib, significantly extends survival in advanced, trastuzumab-resistant breast cancer patients (483).

By age 70, 50-65% of women with deleterious mutations in BRCA1 and 40-57% of women with deleterious mutations in BRCA1 develop breast cancer (78). In sporadic cases of basal-like breast cancer, BRCA1 and BRCA2 are downregulated by either promoter methylation or transcriptional inactivation (61, 62). BRCA1 and BRCA2 are critical components of DNA double strand break repair. Poly(ADP-ribose) polymerase 1 (PARP1) is an essential to DNA single strand break repair. Failure of DNA single strand break repair results in double strand breaks during DNA replication. Therefore, PARP1 inhibitors result in the accumulation of double strand breaks. In tumors with complete BRCA1 and BRCA2 loss double stranded breaks cannot be efficiently repaired resulting in cell death. Cell death induced by the combination of two inactivating events is refered to as synthetic lethality(484-487).

The success of treating breast cancer using targeted therapies is unprecedented among the major forms of cancer. Therapeutic resistance remains a problem in a subset of patients treated with targeted therapies therapy. The promise of targeted therapies is high efficacy coupled with relatively mild side affects. In order to be successful, targeted therapies must be tailored to the patient population that will receive the greatest benefit, using predictive markers. Future advances in the clinical application of DNA sequencing technologies promises to tightly couple the mutational spectrum with appropriate therapeutic modalities. Significant hurdles to the application of personalized medicine exist. Distinguishing causal/driving mutations in the cancer genes from random, passenger mutations can be challenging.

Unmet Clinical Needs

Currently, about one-third of women diagnosed with invasive breast cancer will die from the disease. Major areas of unmet clinical need include: 1) the identification of susceptible populations who could benefit from increased surveillance and the application of cancer prevention strategies, 2) highly sensitive and specific diagnostic technologies capable of discerning tumors with a high likelihood of progressing, 3) prognostic biomarkers capable of identifying early stage breast cancer patients at high risk of recurrence and metastasis, 4) predictive biomarkers of therapeutic response

especially capable of guiding the application of targeted therapeutics, 4) therapeutic modalities or biological technologies capable of targeting essential nodes in growth sustaining pathways, metastatic dissemination, and recurrence, and 5) overcome therapeutic resistance.

Summary

Breast cancer is the most frequently diagnosed malignancy and the leading cause of cancer mortality in women worldwide (28). Epidemiological studies demonstrate a major role for hereditary factors in breast cancer susceptibility (36-39). Environmental and physiological factors are also significantly associated with an individual's risk of developing breast cancer, including age, lifetime estrogen exposure, reproductive history, and obesity (100, 103, 104, 121-123).

The development and function of the mammary gland requires extensive remodeling throughout the reproductive lifespan of an individual. Dynamic tissues are inherently sensitive to tumorigenesis. Frequent proliferative episodes expose relatively long-lived mammary stem and progenitor cells to transformation and tumor initiation (261). Gene expression analysis reveals that breast cancer is a heterogeneous disease that can be separated into several subtypes, including luminal A, luminal B, HER2-enriched and basal-like. The intrinsic subtypes of breast cancer demonstrate distinct etiology, progression, and clinical behaviors (291, 427, 429). Sources of breast cancer heterogeneity include cell of origin and the nature of the genetic and epigenetic alterations within the individual tumor (65).

The stromal microenvironment is essential the normal development and function of the mammary gland. Extrinsic control of the cell proliferation and stem cell properties is an important barrier to breast tumorigenesis (6). Changes in the extracellular matrix and non-malignant cell types within the stromal microenvironment are essential to malignant progression (2-7). Until relatively recently, mainstream breast cancer research efforts have concentrated on the relationships between tumor genetics, cancer cell signaling pathways, and cellular properties of the tumor epithelium. From these efforts, therapeutic targeting pathways essential to tumor growth and progression have been developed. Therapeutic targeting of estrogen signaling and HER2 have dramatically increased the survivability of subsets of breast cancer patients (331, 464,

468, 469). Advances in breast cancer screen, diagnosis, surgery, and the administration of chemotherapy have also been critical to the declining mortality of breast cancer patients (30).

Given the tumor suppressive effects of the normal mammary stroma (6) and the importance of an altered stromal microenvironment to breast tumor progression (2-7), essential microenvironmental factors may represent promising therapeutic anti-cancer targets. A critical advantage of targeting the tumor microenvironment are not susceptible is the lack of selective pressures driving therapeutic resistance in tumor cells. In this dissertation, disequilibrium between NE secreted by activated-neutrophils in the tumor microenvironment and its epithelial-secreted inhibitor elafin is explored. The data presented suggests that deregulated NE-activity is an important factor in tumor growth and a therapeutic target in breast cancer.

NEUTROPHIL ELASTASE

Serine Proteases

More than two-percent of the human genome encodes proteases and 18% of sequences deposited in the SwissProt database have been annotated as undergoing proteolysis. Overall, there are 611 proteases in the human genome and 388 non-functional homologs that have lost residues critical to proteolytic activity (488). The sheer abundance of proteases, attest to their importance in biology. Proteases have evolved to function under a variety of physiological conditions; variables include pH and oxidation state. Early biochemical work on proteases, concentrated on defining their catalytic mechanism. These studies identified five distinct proteases classes, aspartic, cysteine, metallo, serine, and threonine. Glutamic proteases have also been identified but are not found in mammals (489, 490).

Serine proteases, characterized by a reactive serine residue at their catalytic core, are one of the largest and most divergent groups of mammalian proteases (489). Classically viewed as unrefined agents of protein catabolism, many serine proteases are now understood to be highly preferential in their choice of substrates. Pioneering work on the activation of trypsinogen and the mechanism of blood clotting revealed that limited proteolysis can result in altered activity and function (491-493). Proteolytic processing has the ability to rapidly and profoundly alter diverse cellular and physiological processes through irreversible post-translational modification of protein structure and consequently molecular function. Accordingly, serine protease activity is tightly regulated under normal physiological conditions by redundant mechanisms; including endogenous inhibitors, compartmentalization, and expression as zymogens. Inappropriate serine protease activity plays an important role in the etiology and/or pathogenesis of many disease states, including cancer.

Neutrophil Elastase

Neutrophil elastase (NE) belongs to the chymotrypsin superfamily of serine proteases. NE has two catalytically active homologs, proteinase 3 (PR3) and cathepsin G (CG), created by duplication of an ancestral protease gene. Azurocidin shares

significant sequence and structural homology with NE, PR3, and CG, but lacks protease activity (494). NE, PR3, and CG are collectively referred to as neutrophil serine proteases (NSPs), due to their predominate expression in the neutrophil lineage. NE and CG are also expressed in mast cells and monocytes, but at comparatively low levels (495). NE is transcribed from the ELA2 gene on chromosome 19p13.3, which is adjacent to the PR3 gene, PRTN3. CG is transcribed from the CTSG gene residing on chromosome 14q11.2. NSPs share a similar gene structure, consisting of five exons and four introns (494, 496).

Neutrophil Elastase Synthesis

NE is normally synthesized in the bone marrow during myeloid-differentiation and packaged into the azurophilic granules of neutrophils, along with PR3 and CG (497). Neutrophils contain four types of granules, azurophilic granules (primary), specific granules (secondary), gelatinase granules (tertiary), and secretory granules. Neutrophil granules are generated sequentially during differentiation. NSPs are abundantly expressed during the promyelocytic phase of neutrophil differentiation corresponding with the generation of azurophilic granules. Conserved PU.1, C/EBP, and c-Myb binding sites within the promoter sequence of NSPs drive their expression in promyelocytes (498). In addition to NSP, high concentrations of myeloperoxidase, azurocidin, bacterial permeability increasing protein, and defensin are contained within the azurophilic granules. The generation of azurophilic granules is limited to the promyelocyte. As promyelocytes differentiate into myelocytes, the NSP expression is downregulated. NSPs are not detected in specific granules, which are packaged in myelocytes (499). The intracellular concentration of NE in neutrophils is in excess 5 mM (500)

Neutrophil Elastase Activation

NSPs are synthesized in an inactive “pre-pro” form containing a signal sequence and a pro-dipeptide (Ser14-Glu15). Prior to being packaged into the azurophilic granules, pre-pro NE is rapidly processed into its active form first by a signal peptidase and then by a cysteine protease, dipeptidyl peptidase I (DPPI or cathepsin C). NE is also processed at the C-terminus by an unidentified protease. C-terminal processing of

NE does not affect protease activity; instead it creates a docking site for adaptor protein 3 (AP3), responsible for protein shuttling NE between the golgi and granule (501). Cleavage of the N-terminal pro-dipeptide of NE by DPPI is a crucial step in NE activation. Following removal of the pro-dipeptide, the free amino group of isoluecine 16 forms a salt bridge with the carboxylate side-chain of aspartate 194 opening the active site to substrates (502-504). Loss of function mutations in DPPI results in the loss of NSP activity, underlying the rare genetic disease Papillon-Lefevre syndrome (PLS). Patients with PLS suffer from periodontal disease, palmoplantar keratosis, and high susceptibility to systemic infection (505).

Neutrophil Elastase Structure and Function

Active NE prefers to cleave next to small, hydrophobic amino acids (P_1 according to the Schechter and Berger notation), especially valine, isoleucine, and alanine. The substrate specificity of NE overlaps with related NSPs (esp. PR3, 60%-nucleotide similarity). However, the distribution of charged amino acids surrounding their active sites endows each NSP with specificity in substrate recognition (506). NE is a neutral serine protease, demonstrating the highest degree of activity at pH 7.0.

As with all members of the chymotrypsin superfamily, NE cleaves peptide bonds using a charge relay system composed of three essential residues, histidine, aspartate, and serine, collectively referred to as the catalytic triad. The residues of the catalytic triad are similarly dispersed throughout the peptide sequence of chymotrypsin-like proteases (i.e. histidine 57, aspartate 102, and serine 195), however they are brought into close physical proximity by the three-dimensional structure of the protease.

The three dimensional structure of NE consists of two highly similar β-barrels (six anti-parallel β-sheets) connected by a linker segment and a C-terminal α-helix. Four disulfide bonds play a critical role in stabilizing the three-dimensional structure of NE. The catalytic residues are located at the junction of the β-barrels. The active site of the NE runs along the β-barrels perpendicular to the catalytic amino acids (507). A substrate binds to the active site of NE with the sissle bond in close proximity to the reactive serine. The –OH group on the serine acts as a nucleophile, attacking the carbonyl carbon of the sissle bond and forming a covalent bond with the substrate. The nitrogen on the histidine residue accepts the -H from the serine –OH and electrons from

the oxygen of the carbonyl carbon, forming a tetrahedral intermediate. Electrons from the peptide bond between the carbonyl and amino groups of the substrate then attack the hydrogen of the histidine, the bond is broken and the N-terminal portion of the peptide is released. The electrons residing on the nitrogen of the histidine reconstitute the bond, forming an acyl-enzyme intermediate. A molecule of water is used to replace the N-terminus of the cleaved peptide, again through nucleophilic attack of the carbonyl carbon, resulting in the formation of another tetrahedral intermediate. Then the electrons from the bond between the serine and carbonyl carbon attacks the hydrogen on the histidine followed by double bonding of the carbonyl carbon to the oxygen, resolving the tetrahedral intermediate, releasing the C-terminal portion of the cleaved peptide, and resetting the active site of the protease (508).

A list of biologically relevant NE substrates is presented in Table 3.

Neutrophil Elastase Substrates

Extracellular Receptors	
NE Substrate	**Biological Significance**
IL-2Rα(509)	Inhibition, Impairment of T-cells
TNFRII (TNFRSF1B)(510)	Reduced TNFα binding and inhibition of response
CXCR1(511)	Inhibition of IL-8 signal transduction, impaired activation of neutrophils
CD2(512)	Inhibition, Impairment of T-cell activation
CD4(512)	Inhibition, Impairment of T-cell activation
CD8(512)	Inhibition, Impairment of T-cell activation
CD14(513)	Inhibition of Toll-like Receptor 4 responsiveness to bacterial LPS
CD40(514)	Inhibition, Impairment of Dendritic Cells
CD43 (siaiophorin)(515)	Enhanced T-cell adhesion
CD80(514)	Inhibition, Impairment of Dendritic Cells
CD86(514)	Inhibition, Impairment of Dendritic Cells
Urokinase Receptor (CD87)(516)	Inhibition, Inability to bind urokinase
Complement Receptor 1(517)	Inhibition, Insensitivity to complement 3b
Granulocyte colony stimulating factor receptor (G-CSFR)(518)	Inhibition, negatively impacts granulopoiesis
Proteinase-activated receptor-1 (PAR1)(519, 520)	Inhibition, prevents thrombin induced activation
Proteinase-activated receptor-2 (PAR2) (519, 521, 522)	Activation of the ERK signaling pathway
Proteinase-activated receptor-3 (PAR3)(523)	Inhibition, prevents thrombin induced activation

Cytokines, Chemokines, Growth Factors, and Immune Modulators	
NE Substrate	**Biological Significance**
Chemokine (C-C motif) ligand 15 (CCL15)(524)	Activation/liberation of circulating CCL15, monocyte activation
Complement 3(525, 526)	Cleavage of C3bi into isoforms similar to C3c and C3d
Complement 5(527)	Cleavage of C5a into isoform similar to C5b
Granulocyte colony stimulating factor (G-CSF)(518)	Inhibition, negatively impacts granulopoiesis
Tumor Necrosis Factor alpha (TNFα)(528-530)	Conflicting results, may degrade or activate TNFα
Vascular endothelial growth factor (VEGF)(531)	Altered affinity for VEGF receptors, stimulates Akt activation and chemotaxis
IL-1β(532)	Activation of Pro- IL-1β
IL-2(533)	Inhibition, Impairment of T-cells migration

	and adhesion
IL-8(534)	Inhibition, reduced activation of NF-κB
IL-18(535)	Inhibition of IFNγ expression
Macrophage inflammatory protein-1α (CCL3)(536)	Generation of isoforms with reduced chemotactic capabilities
Stromal cell-derived factor-1 (SDF-1 or CXCL12) (537)	Inhibition, reduced chemotactic capabilities
Proepithelin/Progranulin (538, 539)	Degraded, causing enhanced activation of pro-inflammatory signaling

Transcription Factors and Modulators of Intracellular Signaling	
NE Substrate	**Biological Significance**
Cut-like homeobox 1 (CUX1)(540)	Generation of a hyperactive p110 isoform
Cyclin E(541)	Generation of isoforms, hyperactive in their ability to activate CDK2
Insulin Receptor Substrate 1 (IRS-1)(542)	Degraded, enhanced PI3K-AKT activation
PML-RARα(543)	Cleavage required for initiation of acute promyelocytic leukemia in mouse model
NF-κB p65(544)	Diminished NF-κB activation
Notch 2 N-terminal Like (NOTCH2NL or N2N)(545)	Inhibition of Notch2 signaling
STAT3 α-isoform(546)	Generation of STAT3 γ-isoform

Adhesion Molecules and ECM Components	
NE Substrate	**Biological Significance**
E-cadherin(547)	Reduced intracellular adhesion
Collagen(548)	Degradation, alteration of ECM composition
Cadherin-5 (Vascular-Endothelial Cadherin)(549)	Reduced adhesion of endothelial cells, permissive of neutrophil transmigration
Intercellular adhesion molecule 1 (ICAM-1)(550)	Reduced recruitment and transmigration of immune cells
Laminins(551, 552)	Generation of bio-active peptides with chemotactic properties
Fibronectin(552, 553)	Generation of bio-active peptides
P-selectin glycoprotein ligand-1 (554)	Reduced recruitment and transmigration of immune cells
Aggrecan(555)	Disruption of proteoglycan structures
Elastin(556)	Degradation, alteration of ECM composition

Proteases and Protease Inhibitors	
NE Substrate	**Biological Significance**
Matrix metalloproteinase-2(557)	Activation of protease activity

Matrix metalloproteinase-3(558)	Activation of protease activity
Matrix metalloproteinase-9 (559)	Activation of protease activity
Merpin α(560)	Activation of protease activity
Elafin(561)	Inability to bind the ECM and loss of protease inhibitor capability
C1-inactivator(562)	Loss of protease inhibitor capability
Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1)(559, 563)	Loss of protease inhibitor capability
Cystatin C(564)	Loss of protease inhibitor capability
α2-plasmin inhibitor(562)	Loss of protease inhibitor capability
Secretory leukocyte peptidase inhibitor (SLPI)(565)	Inability to bind the ECM and loss of protease inhibitor capability

Blood Clotting	
NE Substrate	**Biological Significance**
Von Willebrand factor (vWf)(566)	Release of vWf from endothelial cells
Platelet integrin (αIIβ3, ITGA2B) (567)	Enhanced integrin function, platelet aggregation
Coagulation factor IX(568)	Activation
Coagulation factor V(569)	Increased association with factor Xa
Heparin cofactor II (570)	Inability to inhibit thrombin
Tissue factor pathway inhibitor(571)	Reduced ability to inhibit factor VIIa/tissue factor
Kininogens(572)	Generation of bioactive kinins, enhanced vasodilatation and smooth muscle contraction
Antithrombin-III(573)	Inactivation, inability to inhibit thrombin

Regulation of Blood Pressure	
NE Substrate	**Biological Significance**
Angiotensin-1(574)	Local vasodilatation
Angiotensin-2(574)	Local vasodilatation
Angiotensinogen(574)	Local vasodilatation

Lipid Metabolism	
NE Substrate	**Biological Significance**
Apolipoprotein A-II(575)	?
Apolipoprotein(a)(576)	?

Insulin Signaling	
NE Substrate	**Biological Significance**
Insulin β-chain(577)	?

Insulin-like growth factor binding protein 3 (IGFBP3)(578)	Alteration of IGF binding

Other	
NE Substrate	**Biological Significance**
IgG heavy chain(579)	Immune dysfunction
Actin(580)	?
Pulmonary surfactant-associated protein D(581)	Impaired anti-microbial and immune functions
Ribonuclease L(582)	?

Table 4: Experimentally Confirmed NE Substrates and the Biological Significance of their Modification.

Role of NE in Neutrophils in Inflammation and Immunity

Circulating neutrophils normally have a half-life of only a few hours, however they are constantly renewed from stem cells in the bone marrow and are the most abundant leukocyte in the bloodstream (comprising 60% of circulating leukocytes in a healthy individual) at a concentration of 1.5 to 5 $x10^9$ cells/liter (506). Neutrophils are a critical component of the innate immune system. Neutropenia, an abnormally low number of neutrophils in the systemic circulation, dramatically increases susceptibility to bacterial infections and can lead to life threatening sepsis, evidencing the importance of neutrophils immunity. Neutropenia is a common side effect in patients undergoing cancer chemotherapy and can increase susceptibility to developing systemic infection. Rare genetic diseases, cyclic neutropenia and severe congenital neutropenia commonly harbor mutations to the ELA2 gene, and less frequently in genes involved in the normal trafficking of NE, or genes involved in the transcription of NE. The mutant forms of NE mislocalize and accumulate within neutrophils, however the mechanism by which mislocalized NE results in neutropenia has not yet been defined (501).

In healthy individuals, neutrophils are the first inflammatory cell type to leave the vasculature following infection or wounding. Neutrophil chemotaxis requires the release of chemotactic cues, such as IL-8, complement 5a, leukotriene B4, and N-formyl-methionyl-leucyl-phenylalanine (fMLP), from the inflammatory site. Neutrophils contain four types of granules, azurophilic granules (primary), specific granules (secondary), gelatinase granules (tertiary), and secretory granules. These granules contain a wide rande of antimicrobial factors, protease, and ROS. Also contained within neutrophil granules are number of membrane bound receptors including CD11b/CD18 and fMLP receptor, which are essential to neutrophil activation and chemotaxis. The intracellular mobilization of granules is essential to the activation and immune functionality of neutrophils.(583, 584).

The major physiological function of NE is the intracellular destruction of pathogens, following phagocytosis at sites of infection. Once engulfed by a neutrophil, pathogens are sequestered in a membrane bound vesicle, know as the phagosome. Fusion of the phagosome with a lysosome creates the phagolysosome, triggering the exposure of the pathogen to massive quantities of reactive oxygen species (ROS)

generated by membrane-bound NADPH oxidase. The phagolysosome containing the pathogen then fuses with neutrophil granules releasing NSPs and antimicrobial compounds, which synergies with ROS in the intracellular destruction of pathogens. Consistent with its important role in innate immunity, NE knockout mice deficient in intracellular killing of gram-negative bacteria and are susceptible to infection (500).

Role of Extracellular NE

Non-resolving inflammation and the presence of inflammatory cells in the tumor microenvironment are regarded as essential components of tumor progression (9, 10). Activated neutrophils also secrete NE into the microenvironment through limited exocytosis of azurophilic granules (585). Neutrophils degranulate, releasing NE, in response to a variety of chemokines, cytokines, and bacterial derived chemotactic peptides, including IL-8, TNF-α, C5a, fMLP, and LPS (586) A portion of highly cationic NE associates with the neutrophil cell surface through electrostatic interactions with negatively charged proteoglycans (587, 588). Neutrophils are the first responders of the immune system and secreted NE is an important component of the early anti-microbial and inflammatory responses. In this context, NE is essential to the formation of neutrophil extracellular traps NETs, complexes formed by the extracellular association of NSPs, antimicrobial peptides, chromatin DNA, and histones. The high concentration of NSPs associates with NETs allows for extracellular killing of microbes and digestion of virulence factors. NE also participates in extracellular ECM remodeling, cleaves cell adhesion molecules, and alters inflammatory/growth factor signaling networks, contributing to the immune response, resolution of infection, and wound healing (589).

Extracellular NE and Cell Signaling

NE promotes activation of toll-like receptor 4 (TLR4), inducing NF-κB activation mediated by MyD88/IRAK/TRAF-6, and ultimately the expression of inflammatory cytokines such as IL-8 (590). The epidermal growth factor receptor (EGFR) may also play a role in the NE-induced activation of pro-inflammatory signaling. Following cleavage by NE the MMP, meprin α, is activated leading to the activation of pro-TGFα, which in turn results in activation of EGFR (560). Immunoprecipitation studies found an association between TLR4 and EGFR following NE simulation and is

essential to NE-induced IL-8 expression (560). NE can also activate the EGFR through the liberation of latent growth factors trapped by the ECM or adhered to the cell membrane (506). Proteinase activated receptor 2 (PAR2) belongs to a family of G-coupled protein receptors that are activated following cleavage of their N-terminal extracellular domains. Several proteases cleave PAR-2, including NE, which activates ERK signaling (521). NE also cleaves PAR-1, inhibiting its activation by thrombin (520) The chemokine stromal cell-derived factor1α (SDF-1α or CXCL12) and its receptor CXCR4 play a critical role in the trafficking of lymphocytes. Cleavage of SDF-1α and CXCR4 by NE inhibits their activation (591). Progranulin (or pro-epithelin), suppresses inflammation by modulating neutrophil activation and preventing the release of NSPs and reactive oxygen species (ROS). NE and PR3 were found to cleave progranulin *in vitro.* In mice deficient in both PR3 and NE, demonstrate a blunted inflammatory response and progranulin accumulation following inflammation induced by immune complexes (538).

Imbalance between NE and its Inhibitors in Inflammatory Disease

Following physiological inflammation, serine protease inhibitors rapidly quench NE activity, inflammation resolves, and tissue homeostasis is restored (506). However, in disease states characterized by chronic or excessive inflammation and neutrophil accumulation, such as emphysema, chronic obstructive pulmonary disease (COPD), (11) and inflammatory bowel disease (IBD) (15), an imbalance between NE and its inhibitors results in symptomatic tissue destruction, perpetuates inflammation, and is critical to disease pathogenesis (11). Deregulated NE activity has been implicated in a variety of inflammatory disease states. NE dramatically alters the immune response by proteolytically altering or degrading a wide rand of cytokine, chemokines, angiogenic factors, growth factors, complemtent, and cell surface receptors.

In animal models, pharmacological inhibition of NE reduced granulocyte accumulation and microvascular dysfunction following ischemia-reperfusion of skeletal muscle (592), prevents the progression of acute lung injury following endotoxin inhalation (593), and decreased the severity of collagen-induced arthritis (594). DPPI knockout mice, deficient in the activation of NSPs, are also protected from experimental arthritis, demonstrating a local decrease in inflammatory makers (595). NE knockout

mice are protected from development of the autoimmune disease, bullous pemphigoid characterized by subdermal blistering (596). Chronic inflammation and the recruitment of neutrophils to the adipose tissue and liver play an integral role in insulin resistance. NE knockout in obese mice fed a high fat diet reduced inflammation, improved glucose tolerance, and enhanced insulin sensitivity compared to wild-type littermates (597). Taken together these studies suggest a prominent role for NE in the pathogenesis of chronic inflammatory disease.

COPD is characterized by limited airflow within the lungs and progressive loss of the lung epithelium. The airways of healthy individuals is sterile, however in patients suffering from COPD bacteria are able to colonize the lung and stimulate massive neutrophil infiltration. COPD is a significant and growing cause of morbidity and mortality within the population. NE is believed to be critical to the degradation of the ECM and destruction of the lung parenchyma. Treatment of COPD patients with NE-inhibitors has been hypothesized to improve lung function. NE inhibitors, such as AZD9668, have been developed for the treatment of COPD. Limited phase one and two studies have shown that AZD9668 is well tolerated, however it did not significantly enhance lung function (598).

Role of Neutrophil Elastase in Tumorigenesis

In the tumor microenvironment the majority of NE is contributed by activated neutrophils. In breast cancer patients, high levels of NE are prognostic of poor overall, metastasis-free, and disease-specific survival, and are predictive of resistance to therapy, suggesting a possible role for neutrophils and NE in tumor progression (12, 599-601). High levels of NE are also found in the tumors of patients with bladder cancer, lung cancer, and pancreatic cancer (12, 601-605). A recent study demonstrates that NE knockout in the *loxP*-Stop-*loxP* K-ras^{G12D} mouse model of lung cancer severely limits tumor growth and proliferation, providing direct *in vivo* evidence of a role for NE in tumorigenesis (542). Sivelestat, a pharmacological inhibitor of NE, was able to reduce the proliferation, motility, and chemotaxis of the pancreatic cancer cell line Capan-1 *in vitro* (603). In a mouse xenograft model of non-small cell lung cancer using the EBC-1 and PC-1 cell lines, sivelestat attenuates proliferation and metastasis (606). Sivelestat also inhibited spontaneous metastasis of EBC-1 xenograft

tumors (607). Beige mice are deficient in NE (608). A mutant strain of NE-deficient mice was created by crossing beige mice with SKH 1 hairless mice. Following ultraviolet irradiation, NE-proficient mice developed 10 tumors per mouse after 20-weeks, while NE-deficient mice developed only 0.4 tumors per mouse over the same period. In the same system, benzopyrene exposure resulted in the formation of 7 tumors per control mouse and only 0.2 tumors per NE-deficient mouse. Pharmacological inhibitors of NE, 2,4,6-trinitrochlorobenzene and oxazolone were able to attenuate the development of skin tumors following ultraviolet irradiation (609). NE knockout in the *loxP*-Stop-*loxP* K-ras^{G12D} mouse model of lung cancer was able to severely limits tumor growth and progression compared to NE-proficient mice (542).

Extracellular NE

The conventionally understood role of NE in tumor progression is largely extrapolated from the contributions of extracellular NE to chronic inflammatory disease, particularly emphasizing the importance of NE in tumor cell invasion and metastasis through ECM degradation and the cleavage of adhesion proteins (610, 611). NE has the ability to cleave the majority of ECM element and is known to activate a number of other tissue degrading proteases including MMP-2, MMP-3, and MMP-9 (559) (612, 613). NE also cleaves adhesion molecules including E-cadherin (550, 614, 615).

Intracellular NE

Several recent studies demonstrate a novel functional context for NE in directly altering intracellular signaling. In these studies, the ability of cancer cells to endocytose NE from the microenvironment (542, 585, 616) or inappropriately express intracellular NE (541, 617) promotes proliferation and tumor progression through specific intracellular proteolytic events. The intracellular cleavage of cyclin E by NE generates low-molecular weight cyclin E isoforms (LMW-E) known to hyper-activate cyclin-dependent kinase 2 (CDK2) (541, 618, 619). LMW-E is prognostic of poor overall, metastasis-free, and disease-specific survival in breast cancer patients (620), induces genomic instability and cellular transformation *in vitro* (621, 622), and can drive mouse mammary tumorigenesis in a CDK2-dependant fashion (623, 624).

Intracellular NE also acts through degradation of Insulin Receptor Substrate-1 (IRS-1). Reduced levels of IRS-1 increase phosphatidylinositol 3-kinase (PI3K) activity

through enhanced association with the platelet-derived growth factor receptor (PDGFR), ultimately promoting Akt activation. In human lung adenocarcinoma, NE and IRS-1 expression are inversely correlated, suggesting that NE-mediated degradation of IRS-1 is relevant to human lung cancer progression (542). Through the proteolysis of a repertoire of extracellular and intracellular substrates, NE plays a critical role in deregulation of many processes and pathways known to promote tumor progression.

NE has been implicated in the pathogenesis of acute promyelocytic leukemia (APL). A significant portion of APL patients harbors a fusion protein consisting of the promyelocytic leukemia gene and the retinoic acid receptor alpha (PML-RARα). NE cleaves the fusion protein PML-RARα in both mouse and human APL and is required for disease progression in mouse models of the disease (543).

In acute myeloid leukemia (AML), the p200 form of transcription factor CUX-1 (CCAAT Displacement Protein) is cleaved by NE into a shorter p110 form, which is hyperactive in its ability to stimulate promoter activity. CUX-1 (p110) is known to increase gene transcription associated with proliferation, motility, and invasion. CUX-1(p110) is overexpressed in breast cancer. Mammary specific overexpression of CUX (p110) drives tumor formation and lung metastasis; indicating that the cleavage of CUX-1 by NE may be important in breast cancer (540, 625).

ELAFIN

Discovery of Elafin

Early reports describe a low-molecular weight inhibitor of elastase-like activity in the bronchial mucus of human subjects. Failure to detect this protease inhibitor in the serum of these subjects suggested to the authors that it was produced locally in the lung, a critical distinction from previously identified elastase inhibitors (626, 627). Subsequent studies identified a serine protease inhibitor, termed elafin, in skin lesions associated with the autoimmune disease psoriasis. These reports revealed that elafin is a highly specific and potent inhibitor of porcine pancreatic elastase (PPE), NE, and PR3 activity (13, 628) with significant sequence similarity to previously identified secretory leukocyte protease inhibitor (SLPI) (629). Nearly concurrent identification by several groups and expression as multiple isoforms has resulted in multiple designations, including skin-derived antileukoprotease (SKALP) and elastase specific inhibitor (ESI), however elafin is the consensus nomenclature used in this dissertation (630).

Elafin Structure

Elafin is expressed from the gene PI3 located on the chromosomal region 20q13 in close proximity to several structurally related genes (631). The PI3 gene is composed of three exons separated by two introns. Following translation elafin is 117 amino acids in length. The N-terminal signal peptide is cleaved in the endoplasmic reticulum reducing the length of elafin to 85 amino acids (approximately 9 kDa). Following cleavage of the signal peptide, elafin is secreted via the canonical secretion pathway.

Elafin contains two critical domains: 1) a N-terminal transglutaminase linking domain, referred to as the cementonin, and 2) a C-terminal globular domain stabilized by four-disulfide bonds. This four disulfide bond structural motif first observed whey acidic protein (WAP) an abundant component of milk in rodent and is therefore refered to as a WAP domain (630). The cementonin domain consists of Gly-Gln-Asp-Pro-Val-Lys repeats that serves as a substrate for the enzyme transglutaminase, which catalyzes the covalent cross-linking of elafin to ECM components. Some non-canonical functions of elafin, such as the ability to bind LPS, have been shown to be dependent

on the cementonin-domain (632). The N-terminal region of elafin could not be resolved by crystallography because of its disordered conformation. Circular dichroism and nuclear magnetic resonance (NMR) analyses reveal that in the presence of membrane lipids the disordered N-terminal region adopts a α-helical secondary structure (633). The C-terminal WAP-domain contains the protease inhibitory loop. The WAP-domain of elafin can be released from the cementonin domain by proteolytic cleavage, generating a 57-amino acid form of elafin (approximately 6 kDa). Removal of the cementonin domain does not effect the protease inhibitor activity of elafin, however in the absence of the cementonin domain elafin can no longer bind the ECM (561).

Physical Interaction between Elastase and Elafin

Elafin non-covalently binds to the catalytic cleft of the target protease blocking access of substrates. This mechanism of action differs from serpins, such as α1-antitrypsin. Serpins bind to the target protease and form a covalent linkage, permanently disabling the active site. The crystal structure of the C-terminal globular domain of full-length elafin in complex with PPE has been resolved (634), as well as the NMR structure of elafin alone (635). The conformation of elafin consists of a central β-sheet stabilized by three disulfide bonds. The protease inhibitory loop is connected to the central β-sheet by two external peptide chains and is stabilized by the fourth disulfide bond. The protease inhibitor region is located between amino acids Leu20(P5) and Leu26(P2'). The side chains of the residues making up the inhibitory loop of elafin forms four hydrogen bonds and with the PPE active site, Ser214(S5)-Arg217(S2'). Extensive van der Waals interactions are also formed between the protease inhibitor domain of elafin (P5-P2') and the active site of PPE (S5-S5'). The sissle peptide bond is between Ala24(P1) and Met25(P1'). The Ala24 carbonyl group projects into the oxyanion hole created by nitrogen atoms of Gly193 and Ser195 of PPE, disfavoring catalysis. A secondary site of interaction between elafin Ser48, Cys49, and Ala52 and PPE Asp97 and Val99, further stabilizes the interaction between protease and inhibitor through hydrogen bonding and van der Waals interaction (634, 635).

Oxidation of Ala24(P1) or Met25(P1') reduces the ability of elafin to inhibit PPE, further evidencing the importance of these residues (634, 635). Replacing the Met25(P1') with either a lysine or glycine residue attenuates the ability of elafin to inhibit

NE and PR3, the glycine substitution is completely ineffective at inhibiting NE (636). The WAP-domain of PI3 and the C-terminal WAP-domain of SLPI (two WAP domains) share significant sequence similarity and are believed to have share a common ancestor, however the protease inhibitory domain of SLPI has the ability to inhibit trypsin, chymotrypsin, NE, and cathepsin G, but not PR3. The P1 residue appears to govern specificity; the P1 residue of elafin, Ala24, is too small to interact with the S1 pocket of chymotrypsin. Comparatively, the P1 residue of SLPI, Leu72, is large enough to inhibit chymotrypsin activity (634, 635).

Evolution of WAP-Domain Containing Genes

Fourteen human WAP-domain containing genes are spread over a 683 kb stretch of chromosome 20q13. The clustering of WAP-domain containing genes suggests repeated duplication during evolution. Several WAP-domain containing proteins are encode by genes outside of the 20q13 cluster including WFIKKN, WFIKKN2, and ps20. Some of the WAP-domain containing proteins are known to have protease inhibitory function, including elafin, SLPI, human epididymis gene product 4 (HE4), and eppin, however the majority fail to display anti-protease activity or are uncharacterized (637). Aside from the cysteine residue required for disulfide bonding, very little sequence conservation is observed between WAP-domain containing proteins. Significant functional diversity may exists between WAP-domain containing proteins, for example the porcine sodium-potassium ATPase inhibitor-2 (SPAI-2) has the ability to inhibit an intestinal NA^{+}/K^{+} ATPase (638).

The spectrum of WAP containing proteins is extremely variable between species. Elafin is a relevant example; there is no rodent homolog of human elafin, however there are six-elafin homologs in the porcine genome (639). WAP-domain containing genes have been subject to dynamic change over a relatively short period of evolutionary time. Comparison of the human and chimpanzee genomes identifies the WAP-locus is one of sixteen regions dense in rapidly evolving genes. One reason for this may be that the genes encoding the semen proteins, semenogelin 1 (SEMG1) and semenogelin 2 (SEMG2), are found within the WAP-domain containing gene locus. In primates with polyandrous mating systems the SEMG proteins form a copulatory plug, however in primates with monoandrous mating systems the SEMG contributes to the

viscosity of the semen but does not form a plug. The high rate of SEMG evolution is correlated with differences post-copulatory sperm competition (due to the evolution of monoandrous and polyandrous mating systems) and semen coagulation (640, 641).

Exon shuffling between an ancestral WAP-domain containing gene and a SEMG-like gene is responsible for the chimeric structure (cementonin and globular domain) of trappin family members, such as elafin. The first and third exons of elafin share sequence homology with SEMG proteins, while the central exon containing a WAP domain. Trappins and SEMG proteins are collectively referred to as rapidly evolving substrates for transglutaminase (REST) (642). Elafin is the only trappin in humans, sequence similarity with SLPI is restricted to the WAP domain (630).

The Role of Elafin in Immunity and Inflammation

Elafin Expression

Systemic serine protease inhibitors, such as α1-antitrypsin and α1-antichymotrypsin, are expressed by the liver and distributed throughout the body by the circulatory system. A germline mutation in the SERPINA1 gene (α1-antitrypsin) results in the accumulation of α1-antitrypsin in the liver and deficiency within the circulation, resulting in severe COPD, emphysema, liver cirrhosis, and several other inflammatory diseases (643, 644). Systemic serine protease inhibitors provide baseline control of deregulated protease activity. Elafin and other “alarm-proteases” are expressed at high concentration by epithelial cells, providing local anti-protease coverage where systemic inhibitors may be inadequate (645, 646). Epithelial cells express elafin either constitutively or in response to inflammatory cytokines, such as IL-1β and TNF-α (13, 647). The p38 MAPK, c-JUN, and NF-κB pathways drive elafin expression downstream of inflammatory cytokines (646, 648, 649). The inducible expression of elafin is especially evident in the epidermis. The normal epidermis does not express elafin, except for the keratinizing cells surrounding the sweat duct and hair follicles. However, under inflammatory conditions, associated with the hyperproliferative disease psoriasis or wounding, elafin is highly expressed in the epidermal layer. Elafin expression in the inflamed epidermis is correlated with the amplitude of neutrophil influx (650-653). Larynx, lung, kidney, liver, and urthera are all elafin negative under non-inflammatory

conditions (647). Compared to the related serine protease inhibitor SLPI, elafin demonstrates much greater cytokine-mediated induction, suggesting that elafin has an especially important anti-protease role following inflammatory stimulus (645).

Constitutive elafin expression has also been observed in mucosal tissues, for example the tongue, tonsils, gingiva, epiglottis, esophagus, vagina, and pharynx all express elafin. In addition to a protease inhibitory role, elafin also boast anti-microbial and immune functions, which likely underlie its constitutive expression at mucosal surfaces (647). Interestingly, the fetal tissues including the epidermis express high levels of elafin, which are downregulated at birth (647). Elafin expression by macrophages and γδ T-cells has also been reported in response to bacterial ligands (654). An inflammatory-like reaction is characteristic of tissue remodeling and other normal homeostatic processes. In the human endometrium, elafin is highly upregulated during menstruation correlating with the influx of inflammatory leukocytes and upregulation of inflammatory signaling, suggesting a role for elafin in tissue remodeling and homeostasis (655).

Protease Independent Functions (Non-Canonical)

Elafin plays a significant role in the immune and inflammatory responses. The bactericidal effect of elafin is largely due to its highly cationic charge, which allows elafin to disrupt bacterial membranes. Elafin also possess the ability to bind LPS and modulate the activity of the toll like receptor (TLR) pathway. Adenoviral overexpression of elafin reduces neutrophil infiltration and cytokine/chemokine levels in mice treated with lipopolysaccharide (LPS) (656, 657). Elafin also mediates anti-microbial immunity through interactions with the innate and adaptive immune response. Elafin can opsonize bacteria, activate antigen-presenting cells, and act as a neutrophil chemo-attractant (632, 633). The antibacterial capacity of elafin against both gram-positive and gram-negative strains has been demonstrated *in vitro* and *in vivo* (20, 632, 658) Elafin has anti-fungal activity that could be disrupted by high salt and heparin implicating its cationic charge (659, 660). Elafin is an important component of the mucosal barrier to retroviral infection. Elafin blocks viral attachment, entry, and replication in experimental models (661-664). Commercial sex workers expressing high levels of vaginal elafin are resistant to HIV infection (662, 665). Although not directly tested, the available

mechanistic data suggests that immune functions discussed here are largely independent of the protease inhibitor domain and require the cementonin domain of full-length elafin.

Imbalance between NE and Elafin

Elafin is a critical component of the epithelial barrier to NE-activity. Imbalance between NE and its inhibitors is implicated in the pathogenesis of a wide range of diseases characterized by excessive or chronic inflammation (15). Low levels of elafin and corresponding high levels of NE are observed in the serum of patients suffering from acute respiratory distress syndrome (ARDS) (14). A polymorphism in the elafin gene (Thr34Pro) correlates with a significant reduction in the serum elafin levels and increases susceptibility to ARDS (666). Compared to healthy individuals, the colon mucosa of patients with inflammatory bowel disease (both Crohn's disease and ulcerative colitis) demonstrates reduced elafin expression and high levels of NE activity (15, 667). Analysis of bronchoalveolar lavage fluid from patients with acute lung injury found that elafin levels increased within 48 hours of onset compared to healthy adults. However, elafin levels decrease over time due to degradation by the 20S proteasome cooresponding to increased NE activity (16). Elafin levels are also decreased in cases of bacterial vaginosis and periodontitis (668, 669). Local decrease in elafin expression within the epidermis enhances pustule formation associated with psoriasis and impetigo herpetiformis (670-672). In the subset of inflammatory diseases examined, elafin levels are generally decreased favoring NE.

Consequences of Elafin Overexpression in Mouse Models of Inflammation

Given the important role of NE in regulating inflammation and symptomatic tissue destruction, the possibility exists that the loss of elafin is a critical factor in the pathogenesis of inflammatory disease. This hypothesis has been examined in mouse models of inflammatory disease. Transgenic overexpression or administration of food-grade bacteria engineered to express elafin protects the colon mucosa from tissue destruction associated with dextran sodium sulfate induced experimental colitis (667). Adenoviral deliver of elafin to the lungs of mice significantly reduced acute lung injury induced by pseudomonas aeruginosa infection (20). Transgenic mice overexpressing

elafin in the cardiovascular system demonstrate reduced NE-activity, lower levels of MMPs, and reduced pulmonary hypertension following chronic hypoxia (19). These mice demonstrate reduced mortality, decreased inflammation, and improved heart function after viral myocarditis (18) or myocardial infarction (17). Mice pre-treated with wild-type elafin were resistant to NE-induced tissue destruction and neutrophil aveolisis in an experimental model of emphysema. However, mice pretreated with mutant elafin lacking protease inhibitory function was unable to attenuate the pathogenesis of experimental emphesyma (636). These results indicate that elafin is a critical counterbalance against NE-activity. Permutations in epithelial elafin expression affect the integrity of the anti-protease shield and dramatically alter the pathogenesis of inflammatory disease.

The anti-inflammatory capacity of elafin may extend beyond its anti-protease activity. Elafin directly reduces NF-κB activation in monocytes exposed to LPS through a protease-independent, but otherwise undefined effect on the ubiquitantion of IκB (673). The contributions of these protease independent properties to the anti-inflammatory role of elafin have not been sufficiently examined. Elafin is currently being tested in clinical trials aimed at determining the ability of elafin to modulate post-operative inflammation (674)

Deregulated NE activity drives vascular cell proliferation and intimal thickening following vascular injury. Transgenic overexpression of elafin in the cardiovascular system of mice prevents the proliferation of smooth muscle cells and the accumulation of inflammatory cells following vascular injury (675). Deregulated NE activity may also play a role in proliferation skin lesions associated with the inflammatory disease psoriasis. Treatment of the mouse epidermis with NE results in epidermal thickening, whereas application of elafin can prevent epidermal proliferation(676).

Deregulation of Elafin in Tumorigenesis

Several studies suggest that elafin is downregulated in poorly differentiated squamous cell carcinomas of the skin, head/neck, and esophagus compared to well-differentiated tumors, suggesting a role for elafin downregulation in the development of a poorly differentiated and aggressive tumor phenotype (21, 22, 677). Elafin expression is absent from the majority of breast tumor derived cell lines when compared to normal

HMECs (23). The bZIP transcription factor, C/EBP β, is required for elafin expression in normal HMECs. C/EBP β is expressed as full-length transcriptional activators, termed liver-enriched activating protein 1 and 2 (LAP1 and LAP2), and a truncated repressor, termed liver-enriched inhibitory protein (LIP). C/EBP β, a transcription factor required for mammary gland development and differentiation In the majority of breast cancer cell lines, elafin is transcriptionally downregulated due to overexpression of a C/EBP β dominant negative isoform. Dominant negative C/EBP β is predominately expressed (as a ratio to full-length, activating isoforms) in a large proportion of breast tumors (24). Recent studies indicate that elafin induces growth arrest and/or apoptosis upon re-expression in cancer cell lines; suggesting that elafin possesses tumor suppressive activity (678, 679). In breast cancer xenograft models the introduction of elafin, by intratumoral injection of elafin expressing adenovirus, greatly decreases tumor growth and progression (25).

In HMECs, the expression of elafin is regulated by C/EBP β transcriptional elements. C/EBP β is frequently deregulated in breast cancer patients due to accumulation of the truncated, inhibitory isoform of C/EBP β (24).

Chapter 2: Imbalance between Neutrophil Elastase and Elafin Promotes Breast Cancer Progression

INTRODUCTION

NE, a potent serine protease, is normally synthesized in the bone marrow during myeloid-differentiation and sequestered within the azurophilic granules of neutrophils (504). NE has broad substrate specificity, preferentially cleaving peptide bonds adjacent to small hydrophobic amino acids (506). The major physiological function of NE is the intracellular destruction of pathogens following phagocytosis at sites of infection (500). Activated neutrophils also secrete NE into the extracellular environment through exocytosis of azurophilic granules. In this context NE has important roles in the anti-microbial, inflammatory, and wound healing responses. Following normal inflammation, serine protease inhibitors rapidly quench NE activity and inflammation resolves (506). Imbalance between NE and its inhibitors is implicated in the pathogenesis of a wide range of diseases characterized by excessive or chronic inflammation (680). Pharmacological inhibitors of NE are under development for the treatment of symptomatic tissue destruction in chronic inflammatory lung diseases (11).

The conventionally understood role of NE in tumor progression is promotion of cell invasion and metastasis through extracellular matrix degradation and the cleavage of adhesion molecules (610). However, several recent reports demonstrate a role of NE in the activation of intracellular signaling pathways during early tumorigenesis. NE was shown to enhance phosphoinositide 3-kinase (PI3K) activity following the degradation of insulin receptor substrate-1. In the *loxP*-Stop-*loxP* K-ras^{G12D} mouse model of lung cancer, NE knockout reduces PI3K activity and severely limits tumor growth (542). NE has also been implicated in cleavage of cyclin E into low-molecular weight isoforms capable of hyperactivating cyclin-dependent kinase 2 and inducing tumor formation in mouse models (541, 624). NE activates intracellular signaling through cell surface receptors, including toll-like receptor 4 (TLR4) (590), proteinase-activated receptor 2 (PAR2) (521), and epidermal growth factor receptor (EGFR) (681), either directly

through proteolysis of the extracellular domain or indirectly through the liberation/activation of latent ligands (506).

Elafin is an endogenous inhibitor of NE and the highly related serine protease proteinase 3. Epithelial cells express elafin either constitutively or in response to inflammatory cytokines (13, 647). Imbalance between NE and elafin has been observed in many diseases, including acute respiratory distress syndrome (14), inflammatory bowel disease (15), and acute lung injury (16). In mouse models, overexpression of elafin diminishes tissue destruction associated with experimental colitis (15), protects against acute lung injury (20), reduces pulmonary hypertension following chronic hypoxia (19), and improves heart function after viral myocarditis (18) or myocardial infarction (17). Permutations in epithelial elafin expression affect the integrity of the antiprotease shield and dramatically alter the pathogenesis of inflammatory disease.

Compelling evidence suggests that disequilibrium between NE and its inhibitors is a critical component in the pathogenesis inflammatory diseases. Inflammation and the presence of leukocytes in the tumor microenvironment are regarded as essential components of malignant progression (9). Tumor-associated neutrophils (TAN) are the major source of NE in the tumor microenvironment. In breast cancer patients, high levels of NE are prognostic of poor overall (OS), metastasis-free, and disease-specific survival (12).

Several studies suggest that loss of elafin-mediated control of NE activity may also be a feature of malignant growth. Elafin is downregulated in poorly differentiated squamous cell carcinomas of the skin, head/neck, and esophagus compared to well-differentiated tumors (21, 22). Subtractive cDNA hybridization identified elafin downregulation in breast cancer cell lines compared to HMECs (23). The transcription factor, C/EBP β, is required for elafin expression in normal HMECs. C/EBP β has critical roles in the regulation of cell proliferation and mammary gland development (24, 682-684). In the majority of breast cancer cell lines, elafin is transcriptionally downregulated due to overexpression of a C/EBP β dominant negative isoform. Dominant negative C/EBP β is predominately expressed (as a ratio to full-length, activating isoforms) in a large proportion of breast tumors, suggesting that elafin is downregulated in these breast tumors (24). However, no published study has comprehensively investigated the expression of elafin during breast tumor progression.

HMECs as an *In Vitro* Model System

No direct homolog of elafin exists within the mouse genome. Therefore, we utilized primary and immortalized HMECs as a model system to interrogate the role of elafin in the mammary epithelium (685, 686). HMECs are derived from freshly disassociated reduction mammoplasty or mastectomy tissue specimens. In some cases, HMEC cell lines have been generated from epithelial cells shed into human breast milk. Primary HMEC cultures contain a heterogeneous population of cells; including luminal epithelial cells, myoepithelial cells, stem/progenitor cells, and fibroblasts (687, 688). In culture, purified HMECs stop proliferating and become senescent after several passages. Early experiments found that HMECs could be immortalized by exposure to mutagenic insult, such as benzo(a)-pyrene and γ-radiation, however the frequency of immortalization was extremely low and the permissive genetic alterations were unknown (689).

A refined model of HMEC growth and arrest in culture, following their derivation from human tissue explants, has lead to the creation of systems for the evaluation of normal breast epithelial cell behavior and carcinogenesis. The study of HMECs has enhanced understanding of the barriers to oncogenesis and the mechanisms by which these barriers are circumvented during tumorigenesis. Primary HMEC cultures exhibit an initial exponential growth phase of between fifteen and twenty population doublings before transiently growth arresting. This stress associated proliferative arrest, termed M0, can be overcome by genetic/epigenetic downregulation of p16 expression. The expression of the viral oncoproteins human papillomavirus (HPV) E7 and SV40-large T-Antigen (SV40-Tag) result in Rb degradation and allow cells to circumvent M0-arrest (689-692).

HMECs bypassing M0 cells are not immortalized and are eventually subject to irreversible replicative senescence (M1), characterized by cell enlargement, cell flattening, increased vacuolization, senescence-associated β-galactosidase activity, low proliferation index, and low apoptotic index (693-695). Telomere attrition causes HMECs to undergo senescence at M1. The finite replicative limit of normal cells is termed the Hayflick limit (694, 696). Inactivation of the p53 pathway can overcome replicative senescence in HMECs, this can be experimentally induced by expression of HPV-E6 or SV40 large T antigen oncoprotein (697, 698). Populations of HMECs

emerging from M1 undergo another 30-60 population doublings eventually reach a state of cell crisis, termed M2. HMECs at M2 are characterized by critically short to non-existing telomeres, have a high proliferation index, an equally high apoptotic index, display polyploidy, and chromosomal abnormalities (695). Somatic cells lack the telomerase enzyme (TERT), which is required for the telomere maintenance (696). The expression of TERT in HMECs that have bypassed M0, allows bypass of both M1and M2 (694, 696, 699)

The immortalization of HMECs requires disruption of the Rb pathway (M0), downregulation of p53 activity (M1), and activation of TERT (M1 and M2). Immortalized HMECs have the capacity for infinite cell division, a hallmark of cancer (10), however they are not capable of transformed, anchorage-independent growth and cannot form tumors in immunocompromised mice. Fully immortalized HMECs can be transformed by the introduction of a single oncogene, such as oncogenic H-Ras (315, 700).

HMECs express high levels of elafin in comparison to breast tumor cells. In cell cycle synchronization experiments, elafin mRNA expression is downregulated in S-phase-enriched cell fractions, indicating a potential role in the regulation of cell proliferation (23). HMECs provide an ideal system to explore the role of endogenous elafin expression in normal epithelial cells.

Hypothesis and Central Findings

The hypothesis tested in this chapter is that imbalance between NE and elafin promotes loss of growth control and is a feature of breast tumorigenesis. In support of this hypothesis, immunohistochemical analysis revealed progressive loss of elafin expression during breast and ovarian tumorigenesis. In HMECs, we showed that elafin was required to maintain quiescence (G0) by opposing the mitogenic effect of NE. Mechanistically, NE-induces activation of the ERK signaling pathway in a TLR4-dependent manner. Our results suggest that imbalance between NE and its endogenous inhibitor, elafin, promotes tumor growth through the mitogenic activation of TLR4. A therapeutic approach designed to correct the imbalance between NE and its endogenous inhibitor, elafin, could limit tumor growth and progression.

MATERIALS AND METHODS

Antibodies

Antibodies Primary antibodies used for immunohistochemistry (IHC), western blot (WB), immunofluorescence (IF), and ELISA were:

Antibody	Species	Clone	Company	Application	Notes
Elafin	Mouse monoclonal	TRAB/2F	Hycult	IHC, WB, IF, ELISA	Epitope: 57 C-terminal amino acids
Elafin	Mouse monoclonal	TRAB/2O	Hycult	IHC, WB	Epitope: Transglutaminase linking domain at the N-terminus
Elafin	Rabbit polyclonal		Hycult	ELISA	
Phospho-Rb site Ser780	Rabbit polyclonal		Cell Signaling Technology	WB	
Total-Rb	Mouse monoclonal	G3-245	BD Biosciences	WB	
p53	Mouse monoclonal	DO-1	Calbiochem	WB	
phospho-p44/42 MAPK (Erk1/2) site Thr202/Tyr204	Rabbit monoclonal	D13.14.4E	Cell Signaling Technology	WB	Used at 1:2000 (WB)
p44/42 MAPK (ERK1/2)	Mouse monoclonal	L34F12	Cell Signaling Technology	WB	
KI-67	Mouse monoclonal	MIB-1	Dako	IF	
TLR4	Rabbit Polyclonal		Santa Cruz Biotechnology	WB	H-80
Actin	Mouse monoclonal	C4, MAB1501	Chemicon	WB	Used at 1:5000 (WB)

Immunohistochemistry

Breast cancer samples were obtained from 793 patients diagnosed with pathologic stage I or II breast cancer (American Joint Committee on Cancer) between 1985 and 2000 and treated by surgical resection at The University of Texas M.D. Anderson Cancer Center (UT MDACC) (701). Samples of normal breast tissue were obtained from reduction mammoplasty and samples of DCIS were also obtained from

surgical resections both performed at UT MDACC. Normal breast and DCIS cases were also obtained from the NIH Cancer Diagnosis Program (progression TMA case sets 3, 5, and 7). Ovarian carcinoma samples were obtained from 213 patients diagnosed with ovarian carcinoma between 1990 and 2007 and treated by surgical resection at UT MDACC. Samples of ovarian cystadenoma, borderline tumors, and normal fallopian tube tissue were also obtained from surgical treatment at UT MDACC. Complete clinical and follow-up data on patients whose tissue samples were utilized in this study were obtained by retrospective review of the patients' files. The Institutional Review Board at UT MDACC approved the use of patient-derived specimens and data and the authors of this study strictly followed ethical guidelines of informed consent and appropriate use of patient data.

The sections were deparaffinized and rehydrated by submerging slides 3 x 5 minutes in Histoclear (National Diagnostics), 1 x 5 minutes in 100% ethanol, 1 x 5 minutes in 90% ethanol, 1 x 5 minutes in 70% ethanol, 1 x 5 minutes in PBS, and 1 x 5 minutes in ddH20. Antigen retrieval was performed for elafin IHC by submerging slides in Antigen Unmasking Solution (Vector Laboratories) at sub-boiling temperature (95^{o}C) for 20 minutes, the slides were then cooled to room temperature (20 minutes) in the solution. The slides were washed 3 x 5 minutes in ddH2O and then submerged in 3% H_2O_2 diluted in methanol for 15 minutes at room temperatures to block endogenous peroxidases. The slides were washed 3 x 5 minutes with 1X PBS and incubated in normal blocking serum from VECTASTAIN Elite ABC Kit (mouse IgG) diluted in PBS for one hour, at room temperature, in a humidified chamber. The slides were washed 3 x 5 minutes with PBS and incubated in the primary antibody diluted in VECTASTAIN blocking serum (antibody concentration = 1:200 for elafin) overnight at 4º in a humidified chamber. The slides were wash 3 x 5 minutes in PBS + 0.1% Tween-20 and then incubated with biotinylated anti-mouse IgG from VECTASTAIN *Elite* ABC kit for 30 minutes at RT. The slides were washed 3 x 5 minutes in PBS + 0.1% Tween-20 and incubated for 30 minutes with ABC solution from VECTASTAIN *Elite* ABC kit. The slides were again washed 3 x 5 minutes in PBS and 2 x 5 minutes in dd$H_2$0 and developed using DAB substrate (Vector Laboratories, following manufactures instructions) for approximately three minutes until maximum color developed under the microscope (elafin TMAs contained sections of spleen as positive controls, once maximum color

developed in these the reaction was stopped by submerging in ddH_2O). The slides were washed in ddH2O and counterstained using Mayer's hematoxylin (Lillie's modification) (DAKO) diluted 1:5 in ddH_20 for 10 seconds. The slides were rinsed in tap-water and dehydrated by submerging 1 x 5 minutes in 70%, 90%, and 100% ethanol followed by 2 x 5 minutes in Histoclear. The slides were coversliped using permount (Fisher Scientific). Evaluation was performed with a Leica DM LM light microscopy using the 40x optical lens. Image acquisition was performed using SPOT Imaging Solutions camera and SPOT Advanced software. Image processing was performed using Adobe Photoshop software (Version 11.0.2).

Cell lines and Culture Conditions

Mortal human mammary epithelial cells (HMECs) 70N, 76N, and 81N were generated from reduction mammoplasty samples (685). Immortalized derivatives 76NE6, 76NF2V, 76NE7, 81NE6, and 81NE7 were obtained from Dr. V. Band (686). These cell lines were generated by transfection of mortal 76N and 81N cells, with DNA from the human papillomavirus (HPV). The 76NE6/81NE6 cell lines were transfected with the E6 gene of HPV-16 and lack p53 due to E6 directed proteasomal degradation (702). The 76NF2V cell line were transfected with a mutant E6 gene (F2V) incapable of degrading p53, but still capable of immortalization (703). The 76NE7/81NE7 cell lines were generated by transfection of the HPV-16 E7 and are devoid of Rb and related pocket proteins due to E7 directed proteasomal degradation (704).

HMEC cell lines were cultured in DFCI-1 media, which consists of α-MEM and Ham's F-12 media (1:1 v/v) (HyClone) supplemented with 1% fetal bovine serum (Atlanta Biological), 0.01 M HEPES buffer, 2 mM L-glutamine, 12.5 ng/mL of epidermal growth factor (EGF), 10 nM triiodothyronine, 0.01 mM ascorbic acid, 2 nM estradiol, 1 μg/mL of insulin, 1 mg/mL hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 0.01 mg/ml transferrin, 2.5 ng/ml sodium selenite, 0.035 mg/ml of bovine pituitary extract and 0.01 mg/ml ciprofloxacin. DFCI-3 (growth factor deprivation) media omits from DFCI-1: bovine pituitary extract, EGF, triiodothyronine, estradiol, insulin, hydrocortisone and substitutes 1% BSA for fetal bovine serum (685, 686). 293T cells for lentiviral packaging, were cultured in DMEM supplemented with 10% fetal bovine serum (Atlanta Biological), 0.01 M HEPES buffer, 2 mM L-glutamine,

and 0.01 mg/ml ciprofloxacin. Tumor cell lines were cultured in α-MEM (HyClone) (HyClone) supplemented with 10% fetal bovine serum (Atlanta Biological), 0.01M HEPES, 2 mM L-glutamine, 0.1 mM non-essential amino acids,1 mM sodium pyruvate and 0.1 mg/mL of ciprofloxacin. Prior to stimulation with NE, the tumor cells were washed three times with PBS and cultured in identically supplemented α-MEM, except 1% BSA was substituted for fetal bovine serum

Cell Synchronization Conditions

Lovastatin (Sigma) was used at a concentration of 10 μM to arrest HMECs in the G1 phase. Aphidicolin (Sigma) was used at a concentration of 10 μM to arrest HMECs in early S phase. Nocodazole (Sigma) was used at a concentration of 5 μM to arrest cells in M-phase.

Lentiviral shRNA Infection

Target specific shRNA was obtained in the GIPZ lentiviral vector system (OpenBiosystems) from the MD Anderson shRNA and ORFeome Core Facility. To generate lentivirus, 70-90% confluent 293T cells in 100 mm^3 culture dishes were co-transfected with 3ug of pCMV deltaR8.2, 3 ug of pMD2.G, and 4.5ug of the pGIPZ shRNA vector, using LipoD293 (SignaGen) transfection reagent (according to manufactures instructions). pCMV deltaR8.2 and pMD2.G were produced by the Didier Trono Lab and made available through the Addgene Repository. Twelve hours after transfection the media was changed to fresh DMEM. The virus containing media was collected 24 and 48 hours later and filtered through a 0.45 μM PVDF membrane. Target cells were infected with the virus containing media in the presence of 8 μg/mL polybrene. The cells were selected in 1ug/ml Puromycin for four to seven days until the population was 100% GFP positive (indicating integration of the shRNA). The shRNA sequences used were:

Rb shRNA1 (V3LHS_340829)- TTGCTATCCGTGCACTCCT;
Rb shRNA 2 (V3LHS_340827)- TTATTTCAGTAGATATCGA;
C/EBPβ shRNA1 (V2LHS_48323)- TTATCATTCATCTGTACAC;
C/EBPβ shRNA2 (V3LHS_371448)- TGAACAAGTTCCGCAGGGT;

Elafin shRNA1 (V2LHS_221931)-TTATCTTGACCTTTAACTG;
Elafin shRNA2 (V3LHS_313908)- TGACCTTTAACTGAAACTT;
TLR shRNA1 (V3LHS_374709)- TACTTTGAATCTTGTTGCT;
TLR4 shRNA2 (V3LHS_374707)- TCTTTACTAGCTCATTCCT;
EGFR shRNA1 (V3LHS_361964)-TTCAGAATATCCAGTTCCT;
EGFR shRNA2 (V2LHS_200678)- TTCCGTTACACACTTTGCG.

Complementation of Elafin Knockdown Cells

Elafin cDNA was obtained (Incyte PI3 cDNA, LIFESEQ1453048, OpenBiosystems) and cloned into the gateway pDONR201 via the gateway BP clonase (Invitrogen) using the following primers:

attB1- Elafin-F:
5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCatgagggccagcagcttcttgatcgtg-3'
attB2-Elafin-R:
5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTActggggaacgaaacaggccatccc-3'

(the gateway recombination sequences are in CAPS and the sequences complementary to the elafin cDNA are in lower case. The resulting elafin pENTR vector was verified by DNA sequencing. To create shRNA resistant elafin expression plasmids, three consecutive codons along the shRNA targeting region were mutated at the wobble position using the quikchange lightning site-directed mutagenesis kit (Stratagene) and the following primers:

shRNA1mut- 5'-ggacaagtttcagttaaagtaaagggcaaagtcaaagcgc-3'
shRNA2mut- 5'-cccgttaaaggacaggtctcggttaaaggtcaagataaa-3'.

The M25G mutation to the protease inhibitor domain of elafin was introduced into the shRNA resistant elafin pENTR vectors again using the quikchange lightning site-directed mutagenesis kit (Stratagene) and the following primer:

elafinM25G-5'-taggggattcaaccctgcgcaccggat-3' (636).

The shRNA resistant elafin pENTR vectors were then cloned into the plenti CMV Blast DEST vector (obtained from the addgene repository) (705) using the LR clonase (invitrogen). The lentiviral particles were packaged using the same method as the pGIPZ lentivirus, see above. Elafin shRNA cells were infected with elafin containing plenti CMV vectors and selected in 20 ug/ml Blastacidin and 1 ug/ml Puromycin until 100% cell kill was achieved in elafin shRNA cells uninfected by plenti CMV vectors, 4-7 days.

DNA Content Analysis

Propidium iodide (PI) staining was performed to examine cell cycle distribution. Following indicated treatments, the cells were harvested by trypsination and washed in PBS. $2x10^6$ cells were fixed by resuspension in ice-cold 70% ethanol and maintained at 4°C for at least 24 hours. The fixed cells were than washed with PBS, and stained overnight at 4°C in 0.5 mL of PI staining solution: 10 µg/mL PI (Molecular Probes), 20 µg/mL ribonuclease A (Sigma) in PBS containing 0.5% Tween-20 (Sigma) and 0.5% BSA (Sigma). The cells were incubated for 30 minutes at 37 °C and filtered through 35µM nylon mesh capped tubes (BD Falcon). PI fluorescence was measured using a BD FACScalibur flow cytometer and analyzed based on DNA content using FloJo software (Version 8).

Preparation of Protein Lysates and Western Blot Analysis

Cells were harvested using trypsin, (for measurement of phosphor-ERK using timepoints less than one hour we harvested cells by scraping them into PBS). The cells were washed in PBS (tissues were not) and resuspended in protease/phosphatase inhibition buffer (PPI); 25 g/ml leupeptin, 25 g/ml aprotinin, 10 g/ml pepstatin, 1 mm benzamidine, 10 g/ml soybean trypsin inhibitor, 0.5 mm PMSF (Phenyl methyl sulfonyl fluoride), 50 mm sodium fluoride, 0.5 mm sodium orthovanadate. Cells were lysed via sonication (Sonicator XL; Misonix Inc.) and the lysates were cleared by centrifugation at 125 000 *g* for 45 min at 4 °C. Protein concentration was determined by Bradford assay (reagents from Biorad). Protein concentration was normalized by addition of PBS and mixed with 2x lammeli buffer (4% SDS, 20% glycerol, 120mM Tris-HCL pH 6.8, and

0.02% bromophenol blue) to a final concentration of 2.5 µg/µl for western blot.

50 µg of protein from each condition being tested was loading into the lanes of a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) gels (15% gels to resolve elafin, 13% gels for ERK and pERK, 10% gels for all other antibodies) and submitted to electrophoresis for 300 volt hours. These gels were then transferred to Immobilon P membrane (Millipore) by electrophoresis for 400 volt hours, at 4°C. The membranes were blocked for 1 hour in BLOTTO (5% nonfat dry milk in TBST; 20 mM Tris, 137 mM NaCl, 0.25% Tween, pH 7.6). Following blocking, the membranes were incubated in primary antibodies typically at a concentration of 0.1 µg/ml in BLOTTO for two hours at room temperature. Following incubation with primary antibody the membranes were washed 3 x 20 minutes in TBS-T and then incubated with goat anti-mouse or anti-rabbit IgG–horseradish peroxidase conjugated secondary antibodies (Pierce) at a dilution of 1:3,000 in BLOTTO for one hour. The membrane was washed 3 x 20 minutes in TBS-T, and developed with the Renaissance chemiluminescence system (Perkin-Elmer Life Sciences, Inc.). The membranes were placed in an autoradiography cassette, exposed to film, and scanned.

Three-Dimensional Culture

The bottom of a six well plate was covered with 500µL of growth factor reduced matrigel (BD Biosciences). HMECs were plated at a concentration of 5000 cells per well in DFCI-1 media supplemented with 2% growth factor reduced matrigel (BD). The media was replaced every three days. The acini were washed once with cold PBS, scraped, and collected in a 15 mL conical tube. Cell recovery solution (BD Biosciences) was added to the Matrigel/acini mixture (1:1 v/v) to digest the matrigel. The acini were washed twice with cold PBS and either embedded in histogel (Thermo) or lysed for western blot. The acini embedded in histogel were fixed in 10% buffered formalin, embedded in paraffin, and sectioned for immunoflourescence (IF) analysis. IF was performed identically to IHC described until after incubation with the primary antibody. The secondary antibody utilized was Alexa fluor 594-conjugated goat anti-mouse (Molecular Probes) and the sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) for 15 min at room temperature, mounted with antifade solution (Molecular Probes), and sealed with clear nail polish (Sally Hansen). An

Olympus FV300 laser scanning confocal microscope (Olympus America) was used to obtain 40x magnification images.

Immunofluorescence

To evaluate the intracellular localization of elafin in HMECs; 5,000 cells were plated on an eight well chamber slide (BD Falcon). Following treatment, slides were fixed with 2.5% paraformaldehyde in PBS (pH 7.5) for 20 minutes at RT, permeabilized in PBS + 0.5 Triton X-100 for 10 minutes at RT, and rinsed three times for 15 minutes each in glycine rinse buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 100mM glycine). Slides were then blocked in blocking buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20, 10% goat serum) for one hour. The slides were incubated in the primary antibody, mouse monoclonal elafin (TRAB/2F; HyCult Biotechnology) and Ki67 antigen (Dako), at a dilution of 1:200 in blocking buffer, overnight at 4^{o}C. The slides were rinsed three times for 20 minutes in wash buffer (blocking buffer without the goat serum) and incubated in secondary anti-body Alexa-Flour 594 conjugated donkey-anti-mouse (Invitrogen). Slides were again rinsed, nuclei were counterstained with 0.5 μg/mL DAPI in PBS, and slides were mounted using Prolong Antifade Reagent (Invitrogen). Imaging was performed using a FV1000 laser confocal microscope (Olympus).

Quantitative PCR Analysis

RNA was extracted from 2 x10^6 cells using the RNAeasy kit (Qiagen) and subjected to on column DNase I (NEB) digestion. The RNA was reverse transcribed (1 μg of mRNA per sample) using the First Strand cDNA Synthesis Kit (Roche). Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems).

Elafin-F-5′-TGGCTCCTGCCCCATTATC-3′
Elafin-R-5′-CAGTATCTTTCAAGCAGCGGTTAG-3’
cFOS-F-5′- TGACTGATACACTCCAAGCGGA -3’
cFOS-R-5′- CAGGTCATCAGGGATCTTGCA-3′
EGR1-F-5′- AGCCCTACGAGCACCTGAC-3′
EGR1-R-5′- GGTTTGGCTGGGGTAACTG -3′

TLR4-F-5'-AGACCTGTCCCTGAACCCTAT-3'
TLR4-R-5'-CGATGGACTTCTAAACCAGCCA-3'
EGFR-F-5'-GCGTCTCTTGCCGGAATGT-3' and
EGFR-R-5'-CTTGGCTCACCCTCCAGAAG-3'
C/EBPβ-F-5'-ACCGGGTTTCGGGACTTGA-3'
C/EBPβ-R-5'-GTTTCGATATCACTGGAG-3'
GAPDH-F-5′-TGTACCGTCTAGCATATCTCCGAC-3′
GAPDH-R- 5′-ATGATGTGCTCTAGCTCTGGGTG-3′.

Sandwich ELISA

Immuno MaxiSorp U96 plate (Nunc) were coated with elafin polyclonal antibody at a concentration of 10 µg/mL diluted in 0.1 M sodium bicarbonate, overnight at 4°C. The plate was washed two times with PBS containing 0.1% Tween-20 and once with PBS between each step. The plate was blocked in 1µg/mL BSA (Sigma) in PBS for four hours at room temperature. 200 µL of conditioned media was added to each well and incubated for 1 hour at room temperature; serially diluted recombinant elafin (calbiochem) was used as a control and to derive a formula for concentration determination. The plate was incubated in mouse monoclonal antibody (TRAF/2O) diluted to a concentration of 50 ng/mL in 0.1µg/mL BSA in PBS for 30 minutes at room temperature. The TRAF/2O elafin antibody detects an epitope on the unfolded N-terminus of elafin and provides greater sensitivity in this assay. The plate was incubated in 50 ng/mL goat anti–mouse IgG HRP conjugated (Thermo) in 0.1µg/mL BSA in PBS for 30 minutes at room temperature. The ELISA was developed using 1-Step Ultra TMB (Thermo) until maximum yellow color developed in the controls with maintenance of linearity, the reaction was quenched with 2M phosphoric acid and absorbance was measured at 450nM.

Luciferase Assay

HMECs were transiently transfected with firefly-luciferase promoter constructs containing the 440 bp proximal elafin promoter (pSPL440), the 440 bp proximal elafin promoter with mutations to the critical C/EBPβ binding sites 4 and 5 (pSPL440m4/5), or the minimal elafin promoter (pSPL94) (all constructs described in Yokota et. al, 2007)

using LipoD293 (SignaGen) transfection reagent (according to manufactures instructions). Cells were co-transfected with CMV-Renilla to control for transfection efficiency. Firefly and renilla luciferase activity were quantified sequentially according to manufactures instructions, using the dual-luciferase reporter assay kit (Promega) and the BD moonlight 3010 luminometer. Firefly luciferase values were normalized to renilla,

Growth Curves

HMECs were plated at a concentration of 2000 cells per well in a 24 well plate. After 24 hours the cells were washed three-times in sterile PBS and cultured in DFCI-3 growth factor depleted media. At each timepoint examined, the cells were harvested via trypsinization and cell number was determined using the trypan blue (Fluka) exclusion test and a standard hemocytometer.

MTT Assay

Cell growth was also measured using the MTT assay in a 96-well plate. HMECs were plated at a concentration of 1000 cells per well. After 24 hours the cells were washed three-times in sterile PBS, cultured in DFCI-3 growth factor depleted media, and treated with NE (Elastin Products) as indicated in the figures. Following treatment, 50 μl of 2.5 mg/mL MTT was added to each well for 4 hours. Following this incubation, all media was aspirated and 100 μl of solubilization solution (20mL 1N HCL, 50 mL 10% SDS, 430 mL isopropyl alcohol) was added to the cells. The plates were placed on a horizontal shaker for one hour and absorbance was quantified using a spectrophotometer (Victor3, Perkin-Elmer) at a wavelength of 590 nm.

Microarray

Total RNA was extracted from 2 x10^6 cells using the RNAeasy kit (Qiagen) and subjected to on column DNase I (NEB) digestion (all performed according to manufactures instructions). RNA integrity was confirmed using an Agilent Technologies Bioanalyzer 2100. The Genomics Core Facility at MD Anderson Cancer Center performed the cDNA labeling, hybridization to the Illumina HT-12 v4 BeadChip, and image acquisition. Raw signal intensities were obtained using the Beadstudio analysis software from Illumina and imported into the lumi Bioconductor package (R version 2.15

and Bioconductor version 2.11). The lumi package was used to perform quality control, background correction by RMA method (implemented in affy package), variance stabilizing transformation, and quantile normalization. To identify differentially expressed mRNAs between the comparative groups, we applied modified two-sample t-tests using limma Bioconductor package. The beta-uniform mixture (BUM) model was used to control false discovery rate (FDR) (706). Differentially expressed genes were identified using fold change greater than 1.5 and a p-value yielding a FDR of 0.10 as cut offs.

Statistics

All experiments were performed in triplicates. The results of each experiment are reported as the mean of experiment replicates. Error-bars represent the standard deviation from the mean. All pair-wise comparisons were analyzed using the unpaired, two-sided, t-test assuming a normal distribution of experimental values. A two-tailed Fisher's exact test was used for contingency table analysis. For all statistical test, a $p<0.05$ was considered significant.

RESULTS

Elafin is Downregulated During Breast Cancer Progression.

Several published studies demonstrate that elafin is expressed at the mRNA level in normal human mammary epithelial cells (HMECs), but not in the majority of breast cancer cell lines (23, 24). Based on these studies, we utilized patient-derived tissue specimens to test the hypothesis that elafin is downregulated during breast cancer progression. Tissue microarrays (TMAs) containing normal breast tissue from reduction mammoplasty specimens (n=24), ductal carcinoma *in situ* (DCIS) (n=54), and invasive breast carcinoma (n=793) were subjected to immunohistochemical analysis using a monoclonal antibody against elafin (Figure 8, A and B) (707). Despite a secretion signal near the N-terminus of the elafin peptide sequence (708), immunohistochemical analysis showed that elafin expression was intracellularly localized (Figure 8A). In order to quantify the expression of elafin in patient samples, we employed a scoring system adapted from Allred et al. that considers both the percentage of positive cells and staining intensity (Figure 9, A and B) (709). Scoring of elafin immunohistochemistry was performed in collaboration with Dr. Cansu Karakas M.D., Dr. Jing Zhang M.D. Ph.D., and Dr. Opoku Adjapong M.D.

Representative photomicrographs of elafin expression in normal breast tissue, DCIS, and invasive breast tissue are presented in Figure 10A. Quantification of elafin expression reveals that elafin is highly expressed in the epithelium of the normal mammary gland in comparison to DCIS and invasive breast tumors. Because all samples of normal breast tissue had an elafin score of 6 (Figure 10B), we defined elafin downregulation as an elafin score of 0-5. Elafin expression was at or above the level in normal breast tissue (i.e., the elafin score was 6-8) in 76% of DCIS lesions but only 17% of invasive breast tumors (Figure 10C). Among the invasive breast tumors, tumors with elafin expression at or above the level in normal breast tissue were more likely than tumors with elafin downregulation to be estrogen receptor and progesterone receptor negative and have higher tumor grade (Table 5). Elafin expression was not associated with trends in recurrence-free survival (Figure 11A) or overall survival (Figure 11B).

We also examined elafin expression by immunohistochemistry during ovarian cancer progression (Figure 12A). Elafin was highly expressed in the epithelium of the normal fallopian tube (n=20), the site of origin for more than half of ovarian carcinomas (710), but elafin expression in normal fallopian tube demonstrated greater variability than was observed for elafin expression in normal breast tissue (compare Figure 9B and Figure 12B). Because most cases of normal fallopian tube had an elafin score of 4 or higher, we defined elafin downregulation as an elafin score of 0-3 (Figure 12B). Elafin expression was comparable to that in the normal fallopian tube in 67% of preinvasive ovarian cystadenomas (n=9), 57% of borderline ovarian tumors (low malignant potential) (n=21), but only 14% of invasive ovarian carcinomas (n=216) (Figure 12C). The distribution of histological subtypes differed significantly by elafin expression: ovarian cancer cases with elafin scores of 0-3 were less likely to be of the endometrioid subtype (Table 6). No association was observed between elafin expression and other clinicopathological parameters (Table 6), RFS (Figure 13A), or OS (Figure 13B).

Overall, these results demonstrated that elafin expression is lost during both breast and ovarian cancer progression.

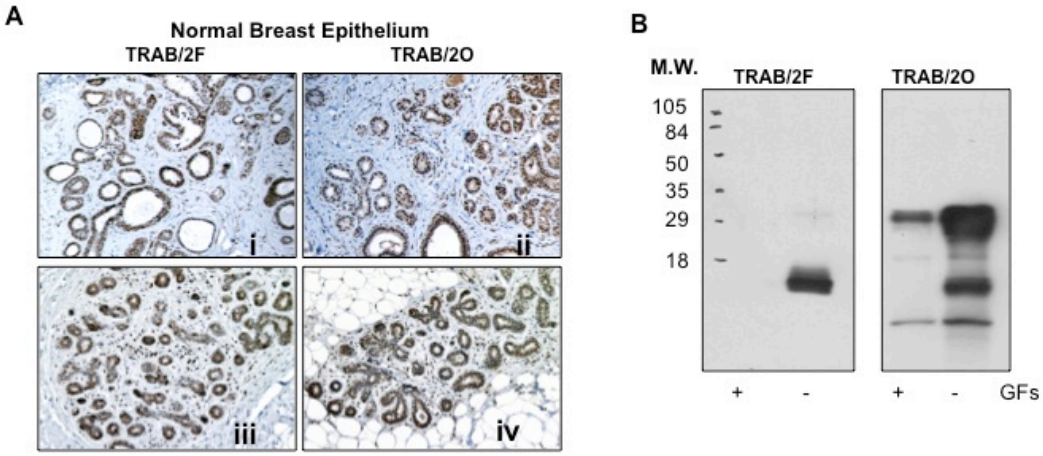


Figure 8: Antibodies for Elafin Immunohistochemistry

(A) Serial sections of normal breast tissue from reduction mammoplasty specimens (i and ii are from the same patient, iii and iv are from another patient) were stained with monoclonal antibodies to the 57 C-terminal amino acids of fully-processed elafin (Hycult, Clone: TRAB/2F) (i and iii) and to the N-terminal transglutaminase linking domain of full length elafin (Hycult, Clone: TRAB/2O) (ii and iv). The intracellular staining pattern was similar for both antibodies. (B**)** HMECs (76NF2V) were cultured in either DFCI-1 growth factor containing media (+GFs) or DFCI-3 growth factor deficient media (-GFs) for 48 hours. Lysates were resolved on a 15% gel in duplicate and subjected to western blot analysis. One half of the membrane was probed with TRAB/2F and the other half was probed with TRAB/2O. TRAB/2F appears to be more specific to elafin than TRAB/2O, which detects several additional proteins of varying molecular weights presumably with transglutaminase-linking domains.

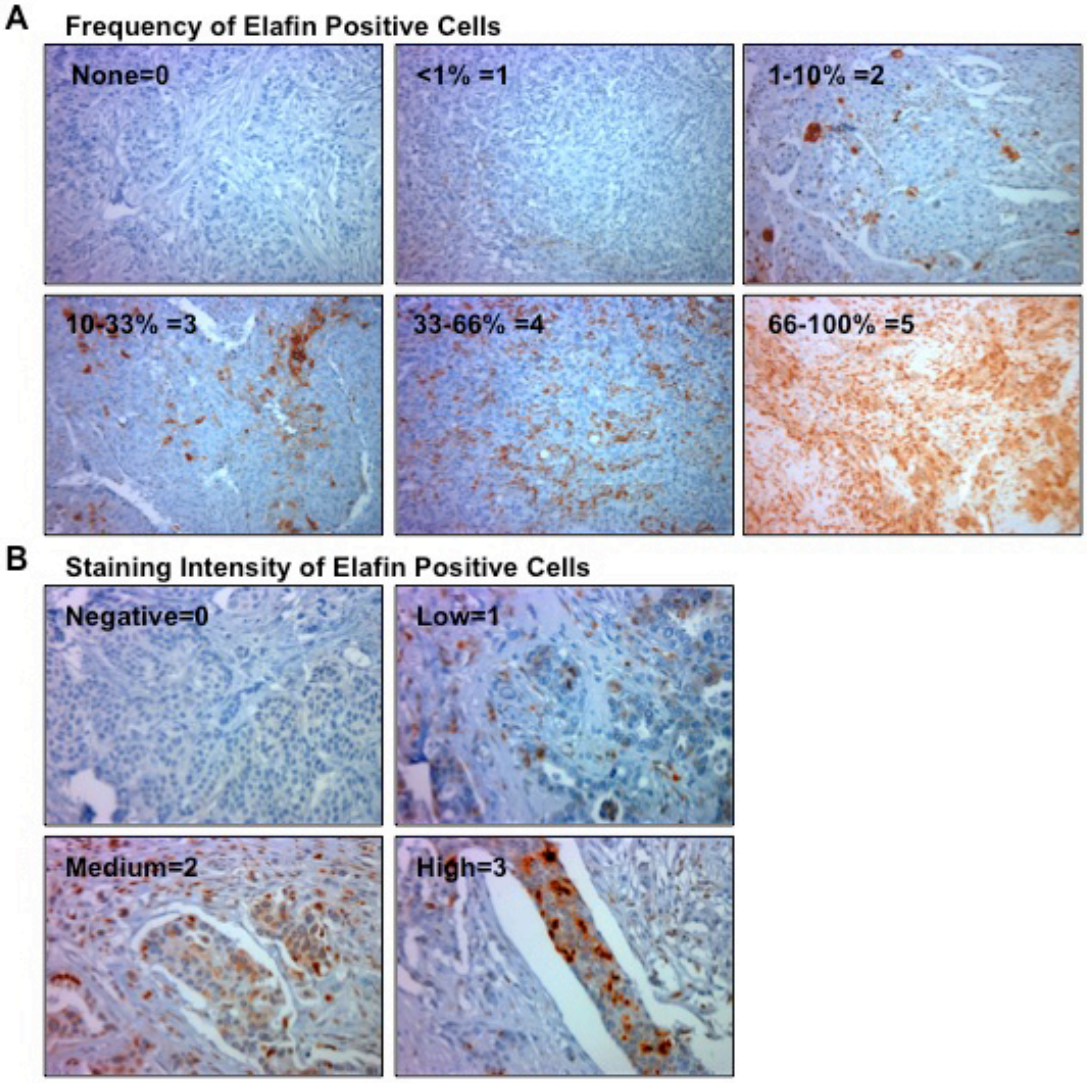


Figure 9: Elafin Immunohistochemistry Scoring System.

(A) The scoring system from Allred et al.,1998 (for the quantification of estrogen receptor positivity in breast tumors) was adapted for elafin IHC scoring. This system consists of a final score (0-8) that is the sum of a frequency score (0-5) and an intensity score (0-3). (A) Representative photomicrographs from invasive breast cancer samples illustrating each frequency score; 0 = 0%, 1 = <1%, 2 = 1-10%, 3 = 10-33%, 4 = 33-66%, and 5 = 66-100%. (B) Representative photomicrographs from invasive breast cancer samples illustrating each intensity score; 0 = Negative, 1 = Low, 2 = Medium, and 3 = High.

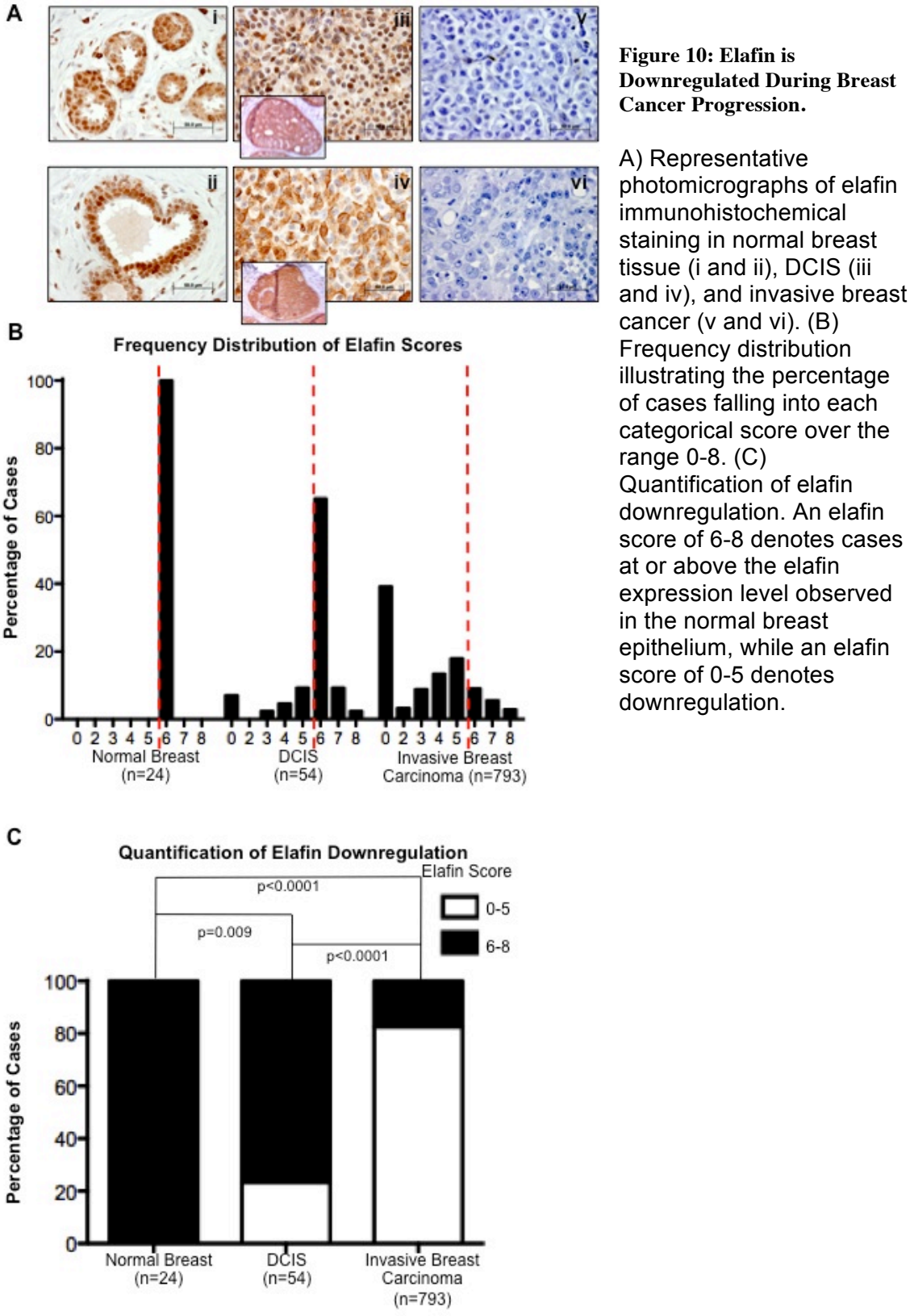


Figure 10: Elafin is Downregulated During Breast Cancer Progression.

A) Representative photomicrographs of elafin immunohistochemical staining in normal breast tissue (i and ii), DCIS (iii and iv), and invasive breast cancer (v and vi). (B) Frequency distribution illustrating the percentage of cases falling into each categorical score over the range 0-8. (C) Quantification of elafin downregulation. An elafin score of 6-8 denotes cases at or above the elafin expression level observed in the normal breast epithelium, while an elafin score of 0-5 denotes downregulation.

Factors	Negative	Positive	p-value
	n=654	n=139	
Age at diagnosis, year			0.9
Mean	53.7	52.4	
Median (range)	55 (25-86)	52 (24-87)	
Stage			0.6
I	200 (31)	35 (25)	
IIA	300 (46)	69 (50)	
IIB	151 (23)	35 (25)	
Unknown	3 (<1)	0	
ER			<0.0001
Positive	474 (73)	72 (52)	
Negative	178 (27)	67 (48)	
Unknown	2 (<1)	0	
PR			0.002
Positive	392 (60)	62 (45)	
Negative	260(40)	77 (55)	
Unknown	2 (<1)	0	
Her-2			0.06
Positive	113 (17)	27 (19)	
Negative	540 (83)	112 (81)	
Unknown	1 (<1)	0	
Grade			0.02
I	61 (9)	11 (8)	
II	332 (51)	51 (37)	
III	215 (33)	71 (51)	
Unknown	46 (7)	6 (4)	

Table 5: Comparison of Breast Cancer Patient and Tumor Characteristics as a Function of Elafin Expression

The correlation between common clinicopathological characteristics of breast cancer patients (age, stage, ER, PR, HER2, and Grade) and elafin expression (positive: elafin score 6-8 and negative: elafin score 0-5) were examined using a fisher-exact test for correlation with elafin expression.

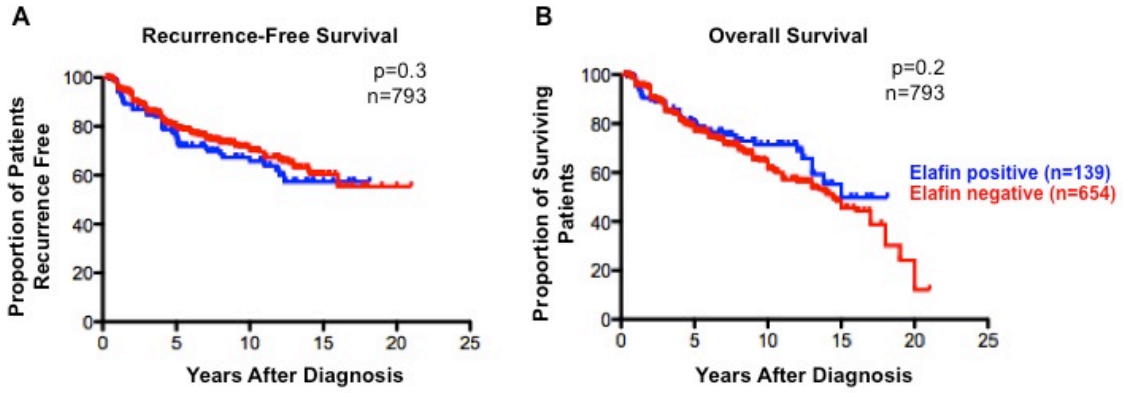


Figure 11: Survival Analysis of Breast Cancer Patients.

(A) Kaplan-Meier analysis of recurrence-free survival in elafin positive and negative breast cancer cases. (C) Kaplan-Meier analysis of overall survival in elafin positive and negative breast cancer cases.

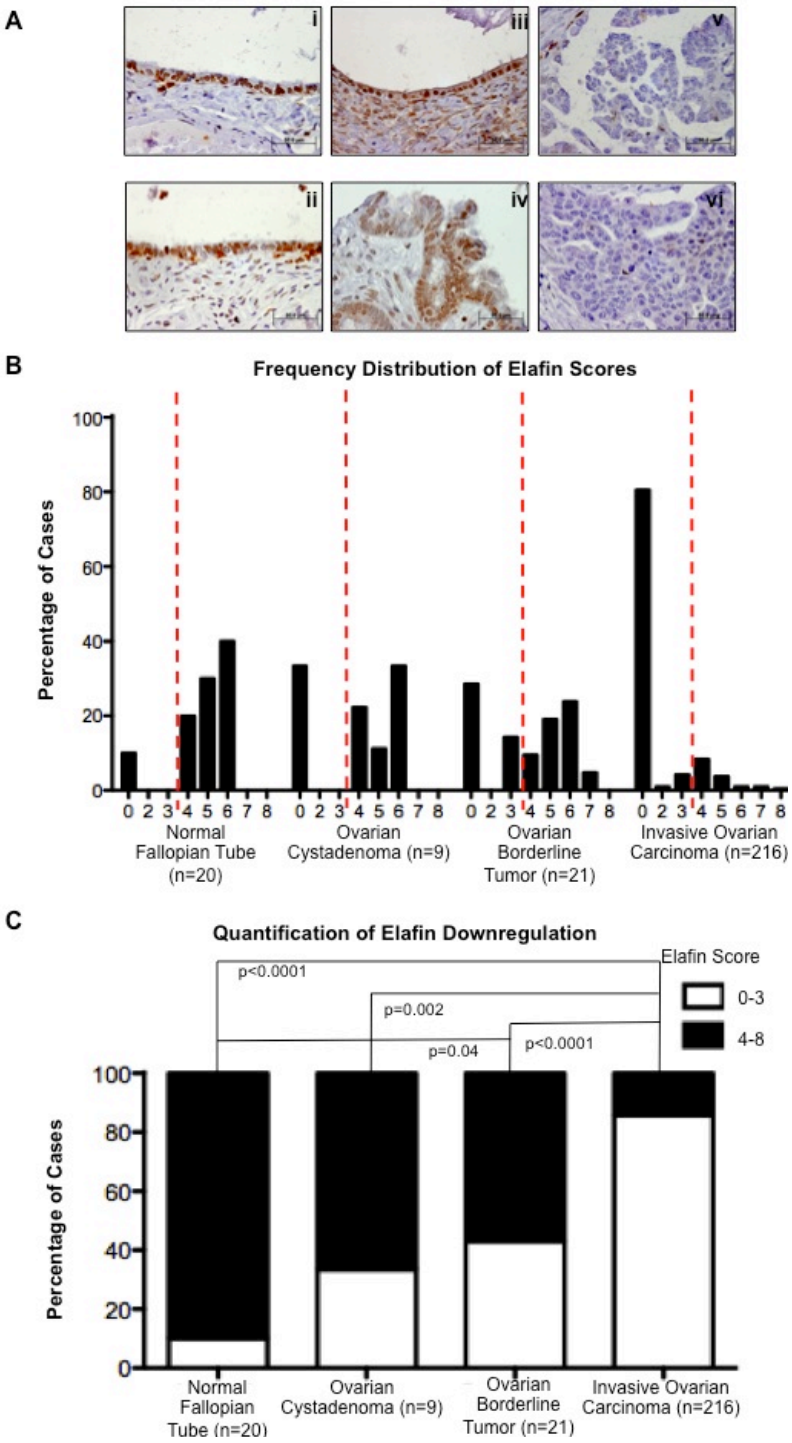


Figure 12: Elafin is Downregulated During Ovarian Cancer Progression.

(A) Representative photomicrographs of elafin immunohistochemical staining in normal fallopian tube (i and ii), nonmalignant ovarian cystadenoma (iii), ovarian borderline tumor (iv), and invasive ovarian carcinoma (v and vi). (E) Frequency distribution as described in B. (F) Quantification of elafin downregulation. An elafin score of 4-8 denotes cases at or above the elafin expression level observed in the normal fallopian tube, while an elafin score of 0-3 denotes downregulation.

Factors	Negative	Positive	p-value
	n=182	n=31	
Age at diagnosis, year			0.43
Mean	58.1	57.1	
Median (range)	58.7 (20-83)	60.7 (27-87)	
Histological Type			0.05
Serous	134 (73)	23 (77)	
Endometroid	20 (11)	1(3)	
Clear Cell	9 (5)	0	
Transitional Cell	4 (2)	2 (6)	
Malignant Mixed Müllerian	8 (4)	0	
Undifferentiated	7 (4)	2 (6)	
Others	1 (<1)	2 (6)	
FIGO stage			0.22
I	20 (11)	1 (3)	
II	14 (8)	1 (3)	
III	119 (65)	20 (65)	
IV	29(16)	9 (29)	
Grade			0.43
Low	16 (9)	2 (6)	
High	166 (91)	29 (94)	

Table 6: Comparison of Ovarian Cancer Patient and Tumor Characteristics as a Function of Elafin Expression

The correlation between common clinicopathological characteristics of ovarian cancer (age, histological type, FIGO stage, and Grade) and elafin expression (positive: elafin score 6-8 and negative: elafin score 0-5) were examined using a fisher-exact test for correlation with elafin expression.

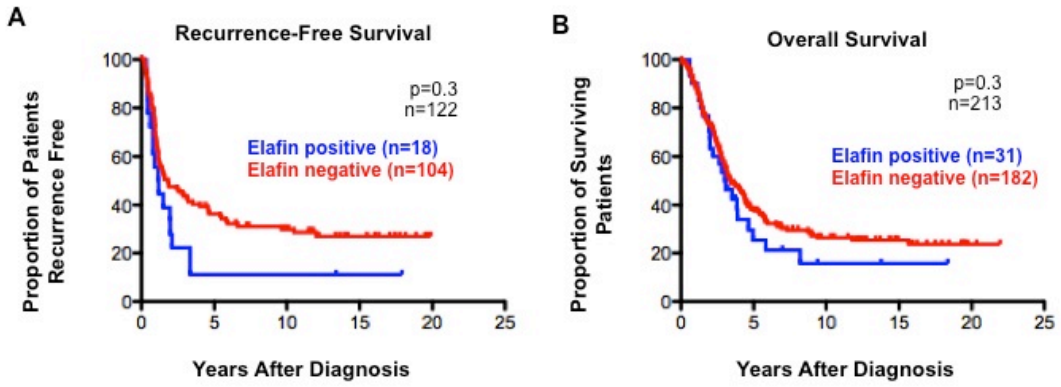


Figure 13: Survival Analysis for Ovarian Cancer Patients.

(A) Kaplan-Meier analysis of recurrence-free survival in elafin positive and negative ovarian cancer cases. (B) Kaplan-Meier analysis of overall survival in elafin positive and negative ovarian cancer cases

Elafin is Upregulated in Mortal HMECs Following Growth Factor Deprivation-Induced Cell Cycle Exit.

Elafin is highly expressed in HMECs and was previously shown to be cell cycle regulated at the mRNA level (23). Given the relevance of cell cycle alterations to early tumor progression, we hypothesized that elafin is required for normal cell cycle control. Growth factor-deprivation induced G0 arrest of immortalized HMECs results in the upregulation of elafin compared to the low levels observed in HMECs asynchronously proliferating in growth factor-containing medium or arrested in the G1, S, or G2/M phase of the cell cycle (Figure 14).

To further explore the expression of elafin in G0-arrested cells, primary (i.e. mortal) 81N, 70N, and 76N HMECs were cultured under growth factor-deficient conditions and harvested at 6-hour intervals for 48 hours. DNA content analysis revealed that HMECs were progressively arrested in G0/G1 phase, and complete arrest was seen between 24 and 36 hours of continuous culture (Figure 15A). Western blot analysis showed upregulation of elafin over the growth factor deprivation time course (Figure 15B), corresponding to the accumulation of HMECs in G0/G1 phase.

We next performed immunofluorescence analysis of Ki67, a marker of cells actively progressing through or arrested within the cell cycle, which is downregulated in G0 cells (Figure 16A). Quantitation of Ki67-positive cells revealed that in growth factor-containing medium, 70-76% of HMECs expressed Ki67, whereas under growth factor deprivation, only 6-10% of total HMECs at 24 hours and fewer than 1% of total HMECs at 48 hours expressed Ki67. This downregulation of Ki67 confirmed that growth factor deprivation of HMECs induces arrest in G0 and not G1. Elafin expression, examined by immunofluorescence, dramatically increased in intensity following growth factor deprivation, and elafin acquired a strongly cytoplasmic localization (Figure 16B).

Quantitative RT-PCR (qPCR) analysis revealed that elafin is highly induced at the mRNA level under growth factor deprivation conditions, increasing 12- to 178-fold by 24 hours and 165- to 240-fold by 48 hours compared to the level of elafin mRNA in HMECs cultured in growth factor-containing medium (Figure 16C). We also examined elafin levels in the conditioned media of 81N, 70N, and 76N HMECs by ELISA. Fresh growth factor-deficient medium was added halfway through the 48-hour time period,

such that all samples of conditioned medium measured in this assay were incubated with cells for 24 hours. The concentration of elafin in the conditioned media of 81N, 70N, and 76N HMECs cultured in the presence of growth factors was 2.6-2.9 ng/mL, compared to significantly higher concentrations of elafin in HMECs cultured in growth factor-deprived medium: 4.0-6.3 ng/mL at 24 hours and 7.1-11.5 ng/mL at 48 hours (Figure 16D).

Luciferase reporter analysis of the elafin promoter revealed that elafin upregulation in HMECs was dependent on C/EBPβ sites 4 and 5, as we previously described (24) (Figure 17A). In the immortalized HMECs 76NF2V, knockdown of C/EBPβ (Figure 17B) rendered cells incapable of elafin upregulation following growth factor deprivation (Figure 17C).

These experiments demonstrated that primary HMECs induced to enter G0 by growth factor deprivation highly upregulate elafin at the mRNA level, resulting in the intracellular accumulation and secretion of elafin.

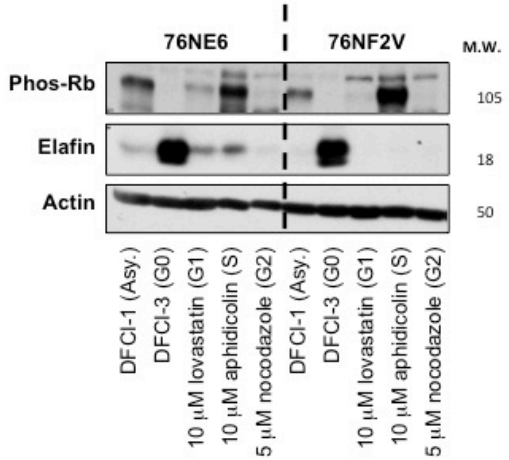


Figure 14: Elafin is Upregulated Following Growth Factor Deprivation

(A) Western blot of phosphorylated Rb (S780) and elafin in 76NE6 and 76NF2V cells cultured in DFCI-1 medium (asynchronous), DFCI-3 medium (arrested in G0), 10 μM lovastatin (arrested in G1), 10 μM aphidicolin (arrested in early S phase), and 5 μM nocodazole (arrested in M phase).

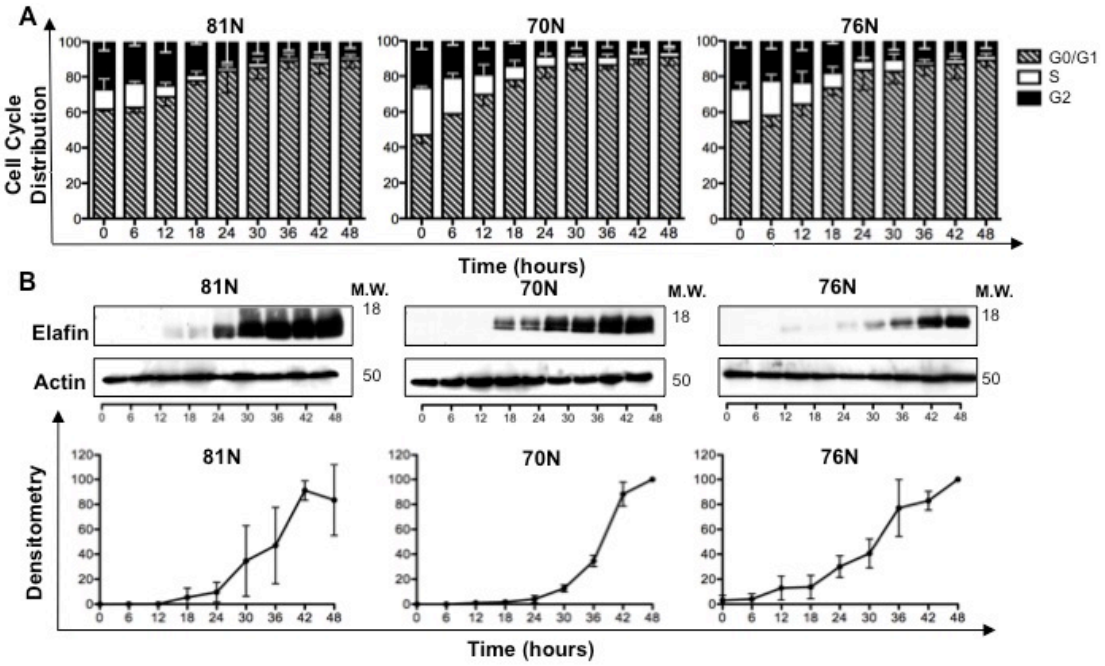


Figure 15: Elafin is Upregulated in Mortal HMECs Following Growth Factor Deprivation-Induced Cell Cycle Exit.

(A-B) 81N, 70N, and 76N HMECs were cultured in growth factor depleted DFCI-3 media and harvested every six hours for 48 hours following growth factor removal (n=3 for each timepoint). (A) Cell cycle distribution determined by DNA content analysis of propidium iodide stained cells, measured by flow cytometry. (B) Representative western blots of elafin expression, actin as a loading control. Elafin western blots were analyzed by densitometry; values normalized to actin and are represented as a percent of the maximum elafin expression

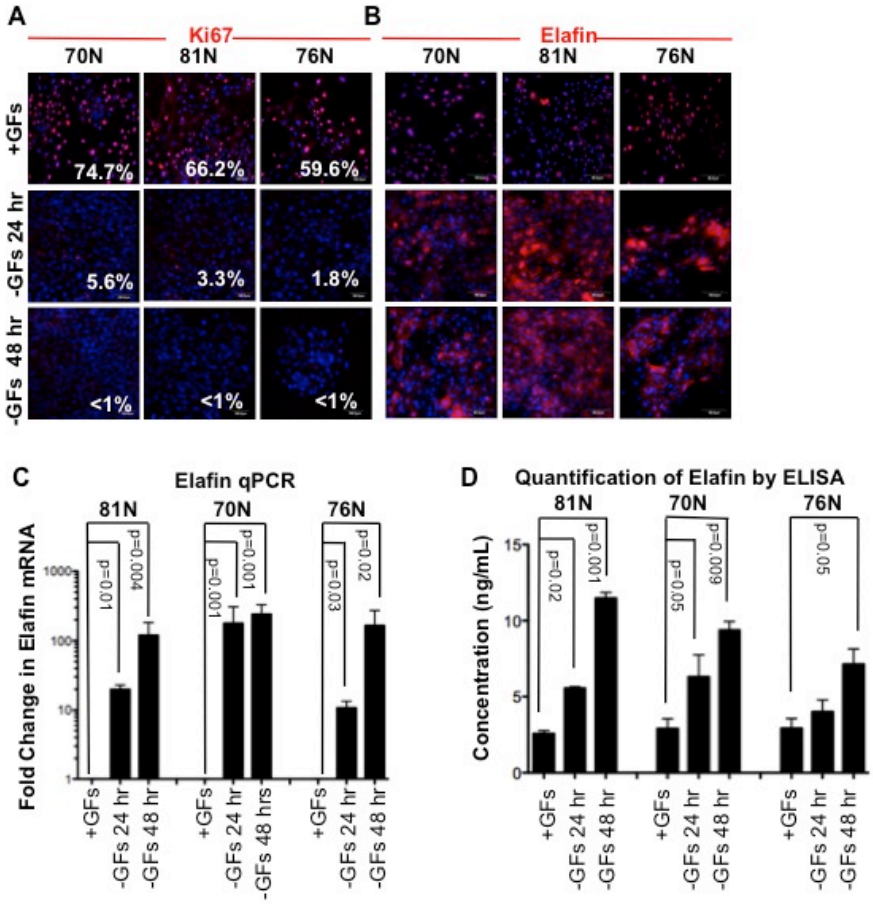


Figure 16: Elafin is Upregulated in Mortal HMECs Following Growth Factor Deprivation-Induced Cell Cycle Exit.

(A,B) 81N, 70N, and 76N HMECs cultured in either DFCI-1 (+GFs for 24 hours) or DFCI-3 media (-GFs for 24 and 48 hours) were examined by immunofluorescence staining for Ki67 (A) and elafin (B) expression. (C) HMECs cultured as in D were examined by quantitative RT-PCR for elafin expression; values were normalized to GAPDH expression and are represented relative to control (+GFs condition). (D) Conditioned media from HMECs treated as in D was examined by sandwich ELISA for elafin levels, concentrations are reported as ng of elafin per mL of media.

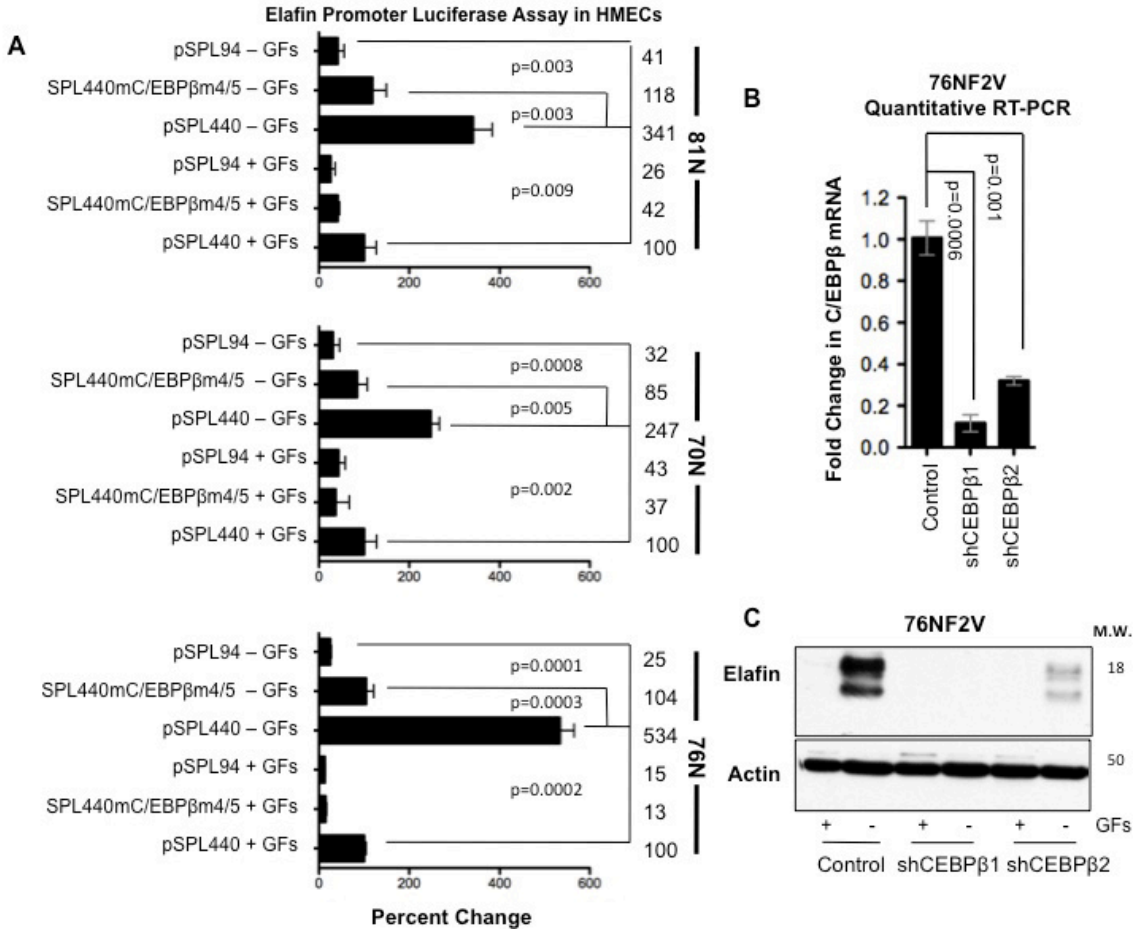


Figure 17: Elafin Upregulation in Mortal HMECs Following Growth Factor Deprivation-Induced Cell Cycle Exit is Dependant on C/EBPβ.

(A) 81N, 70N, and 76N cells were co-transfected with the indicated firefly luciferase elafin promoter construct and CMV-Renilla luciferase and cultured in either DFCI-1 media (+GFs, for 24 hours) or DFCI-3 media (-GFs, for 24 hours). Elafin promoter activity was normalized to renilla and expressed relative to control (pSPL440 +GFs). (B) 76NF2V cells stably infected with pGIPZ lentiviral vectors containing control or two unique, C/EBPβ-specific shRNAs were cultured in DFCI-1 (+GFs) or DFCI-3 (-GFs) media for 24 hours, knockdown was confirmed by qPCR analysis using C/EBPβ specific primers, values were normalized to GAPDH and represented as a ratio to the control shRNA level. (C) Lysates from C/EBPβ knockdown and control 76NF2V in B were subjected to western blot analysis for elafin. Actin, loading control.

Rb-Deficient HMECs are Incapable of Entering Quiescence and Fail to Upregulate Elafin Following Growth Factor Deprivation.

In G0-arrested cells, Rb is required to prevent E2F-induced cell cycle re-entry (711). Immortalized 76NE6 HMECs, which lack p53, and 76NF2V, which express both p53 and Rb (686), progressively arrested in the G0/G1 phase when cultured in growth factor-deficient medium (Figure 18A). However, 76NE7 HMECs, which are deficient in Rb and related pocket proteins (686), accumulated in the G2 phase of the cell cycle when cultured in growth factor-deficient medium (Figure 18A). 76NE6, 76NF2V, and 76NE7 HMECs cultured with growth factors were Ki67 positive (76.3-83.5% of cells). Following growth factor deprivation for 48 hours, only 8.4% of 76NE6 cells and 7.3% of 76NF2V cells were Ki67 positive, while 63.9% of 76NE7 cells remained Ki67 positive (Figure 18B). Maintenance of Ki67 expression in growth factor-deprived 76NE7 cells suggests that loss of the Rb checkpoint eliminates the ability of HMECs to arrest in G0.

Western blot analysis of elafin expression over 48 hours of growth factor deprivation demonstrated elafin upregulation in 76NE6 and 76NF2V cells (Figure 18C). In contrast, 76NE7 cells did not express Rb and did not demonstrate elafin upregulation following 48 hours of growth factor deprivation (Figure 18D). This elafin expression pattern was confirmed by immunofluorescence analysis (Figure 18E). Culture of HMECs on reconstituted basement membrane (matrigel) generates three-dimensional structures resembling mammary acini (316, 712, 713). Immunofluorescence analysis finds the upregulation of elafin in the matrix-deprived, growth-arrested interior of mammary acini formed from 76NE6 and 76NF2V, but not 76NE7 cells (Figure 19)

Following 48 hours of growth factor deprivation, Rb-deficient 81NE7 HMECs failed to upregulate elafin compared to Rb-expressing 81NE6 and parental 81N cells (Figure 20A). 81NE7 could not arrest in G0/G1 phase as indicated by DNA content analysis (Figure 20B). 76NF2V and 76NE6 cells with Rb knockdown were also unable to arrest in G0/G1 phase (Figure 20C) and were incapable of elafin upregulation (Figure 20D) following growth factor deprivation.

Taken together, these results showed that Rb is required for G0 arrest and concomitant elafin upregulation.

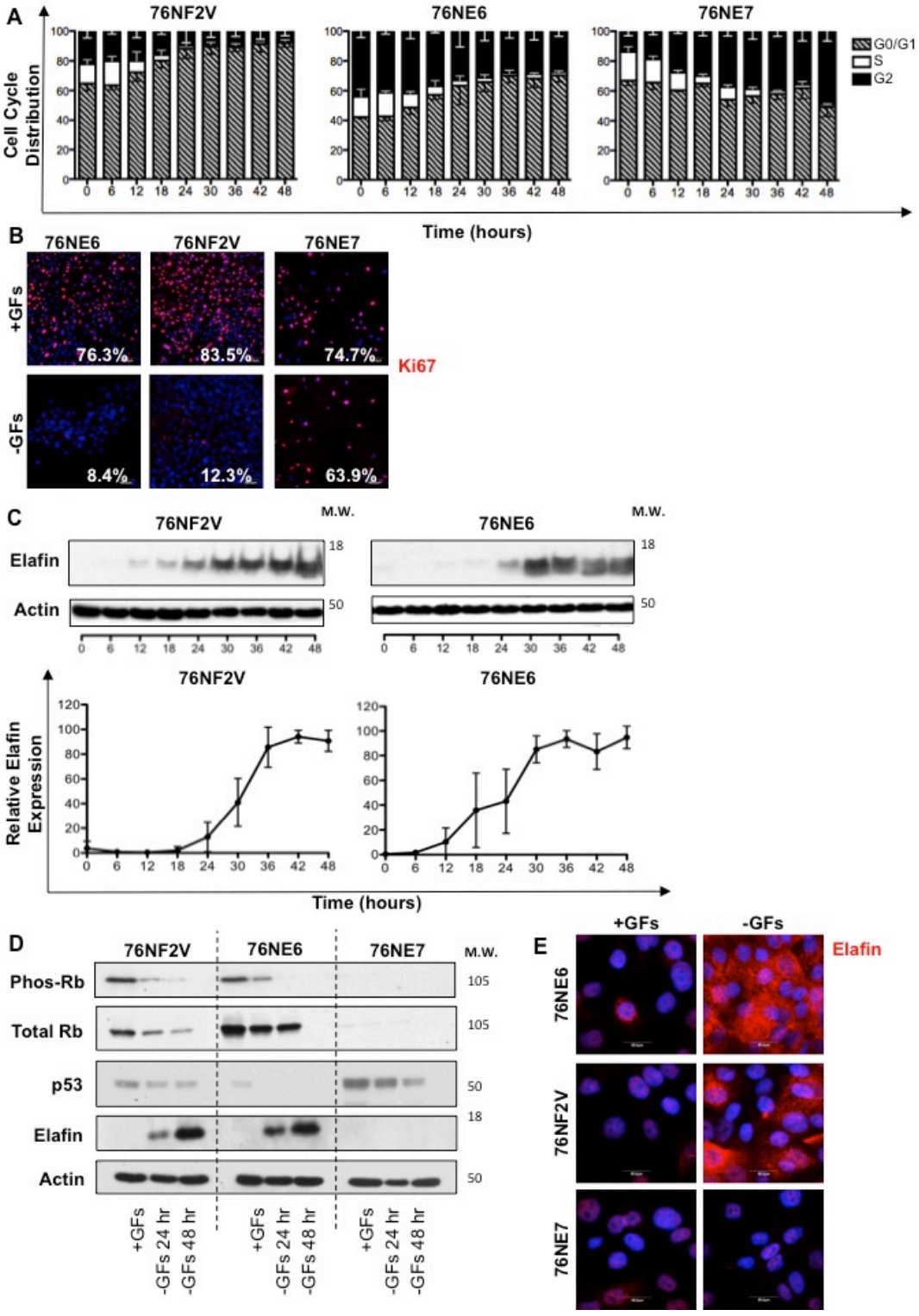

A
76NF2V
76NE6
76NE7
Cell Cycle Distribution
G0/G1
S
G2
Time (hours)
B
76NE6
76NF2V
76NE7
+GFs
-GFs
76.3%
83.5%
74.7%
8.4%
12.3%
63.9%
Ki67
C
M.W.
76NF2V
76NE6
Elafin
Actin
18
50
Relative Elafin Expression
Time (hours)
D
76NF2V
76NE6
76NE7
M.W.
Phos-Rb
Total Rb
p53
Elafin
Actin
105
50
18
+GFs
-GFs 24 hr
-GFs 48 hr
E
+GFs
-GFs
Elafin
76NE6
76NF2V
76NE7

Figure 18: Rb-Deficient HMECs are Incapable of Entering Quiescence and Fail to Upregulate elafin Following Growth Factor Deprivation.

(A-D) 76NE6, 76NF2V, and 76NE7 immortalized HMECs were growth factor deprived as in figure 10A. (A) Cell cycle distribution determined by DNA content analysis of propidium iodide stained cells, measured by flow cytometry. (B) Immunofluorescence staining for Ki67 at the 0 and 48 hour timepoints. (C) Representative western blots of elafin expression (only 76NE6 and 76NF2V shown), actin as a loading control. (D) Densitometry analysis of elafin western blots; values normalized to actin and are represented as a percent of the maximum elafin expression (E) Western blot of phosphorylated Rb (S780), total Rb, p53, and elafin in 76NE6, 76NF2V, and 76NE7 cultured in DFCI-1 (+GFs for 24 hours) or DFCI-3 media (-GFs for 24 and hours), actin as a loading control. (E) 76NE6, 76NF2V, and 76NE7 cells cultured in either DFCI-1 (+GFs for 24 hours) or DFCI-3 media (-GFs for 24 hours) were examined by immunofluorescence staining for elafin expression.

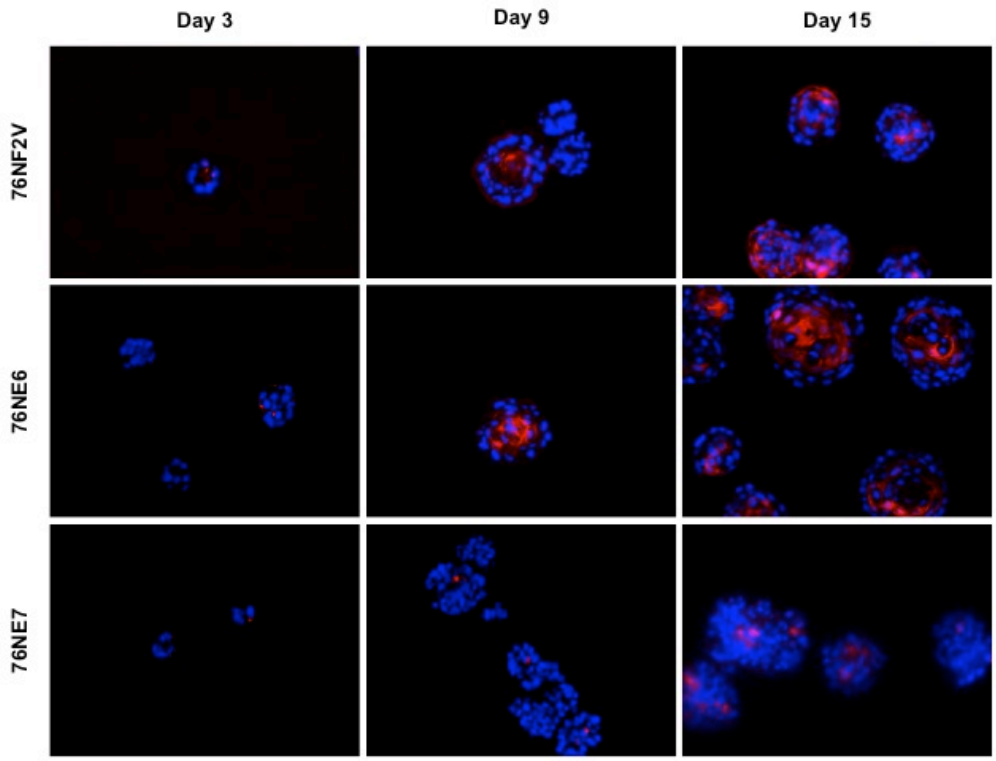
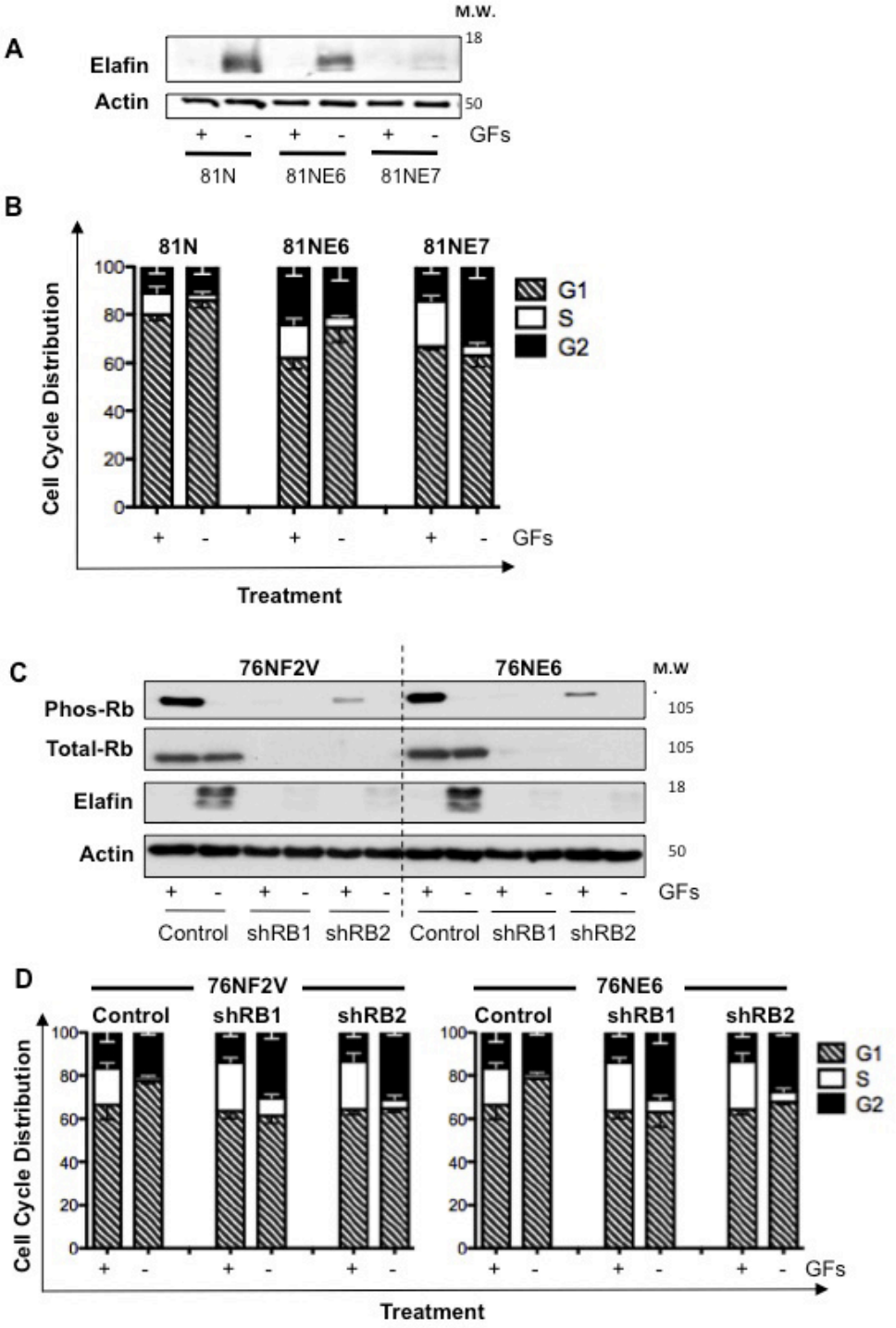


Figure 19: Elafin Expression in Mammary Acini.

(A) 76NE6, 76NF2V, and 76NE7 cells were grown on reconstituted basement membrane (matrigel), acini formed and were harvested at 3, 9, and 15 days; at each timepoint acini were formalin-fixed, paraffin-embedded, and subjected to immunofluorescence staining for elafin expression.


A
M.W.
Elafin
Actin
18
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81N 81NE6 81NE7
B
81N
81NE6
81NE7
Cell Cycle Distribution
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Treatment
C
76NF2V
76NE6
M.W
Phos-Rb
Total-Rb
Elafin
Actin
105
105
18
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Control shRB1 shRB2 Control shRB1 shRB2
D
76NF2V
76NE6
Control
shRB1
shRB2
Control
shRB1
shRB2
Cell Cycle Distribution
100
80
60
40
20
0
G1
S
G2
+ - + - + - + - + - + - GFs
Treatment

Figure 20: Quiescence and Fail to Upregulate elafin Following Growth Factor Deprivation.

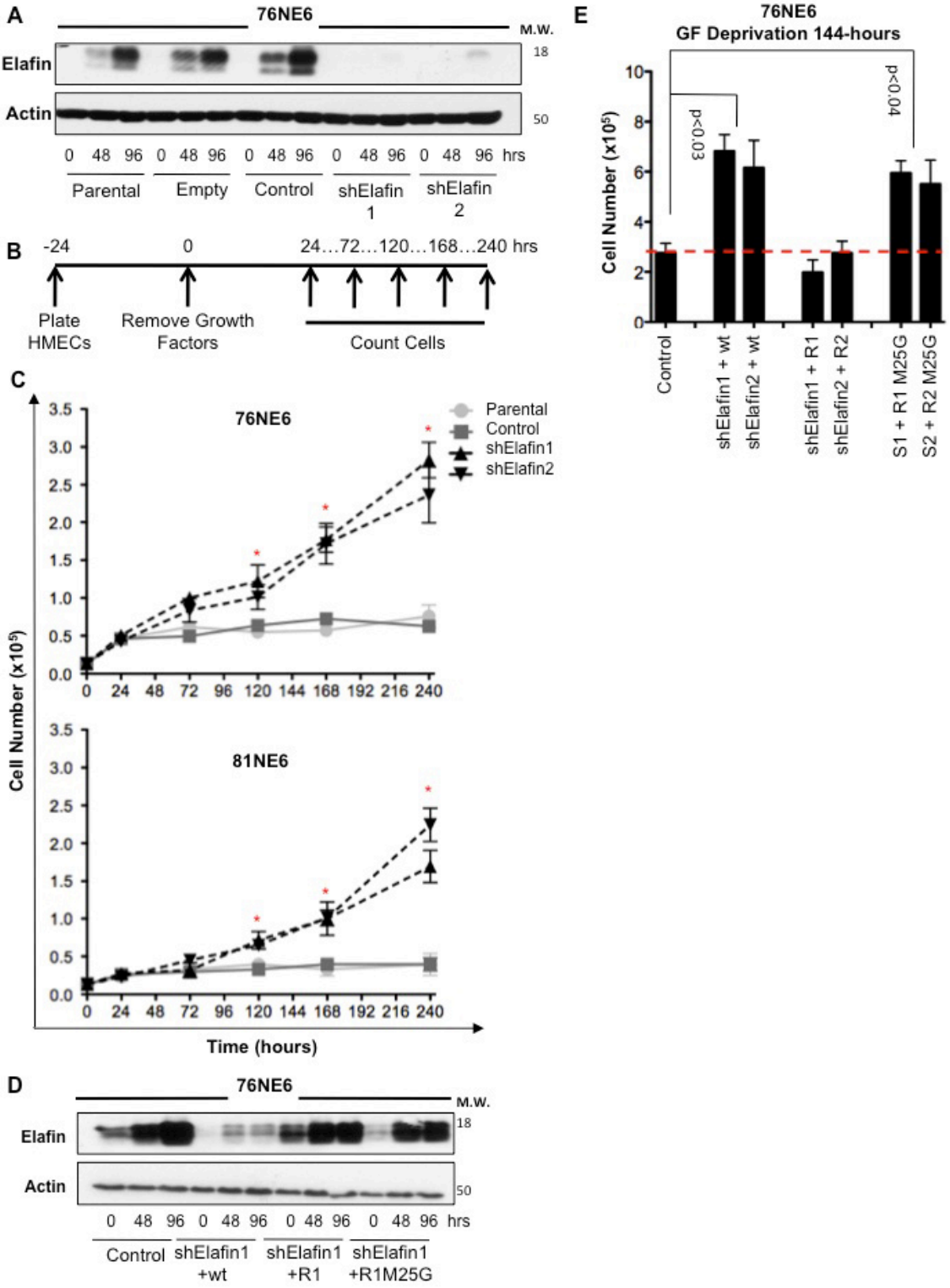
(A,B) 81N, 81NE6, and 81NE7 cells were cultured in either DFCI-1 (+GFs) or DFCI-3 media (-GFs) for 24 hours. 81N cells immortalized by HPV-E6 lack p53 and 81N cells immortalized by HPV- E7 lack Rb and related pocket proteins. (A) Lysates were subjected to western blot analysis for elafin. Actin, loading control. (B) Cell cycle distribution determined by DNA content analysis of propidium iodide stained cells. (C,D) 76NF2V and 76NE6 cells stably infected with pGIPZ lentiviral vectors containing control and two unique, RB-specific shRNAs were cultured in DFCI-1 (+GFs) or DFCI-3 (-GFs) media for 24 hours. (C) Western blot of phosphorylated Rb (S780), total Rb, and elafin. Actin, loading control. (D) Cell cycle distribution was determined DNA content analysis of propidium iodide stained cells.

Elafin Knockdown HMECs Circumvent Quiescence and Proliferate in a Growth Factors Independent Manner.

To examine the role of elafin in maintenance of G0 arrest, we generated 76NE6 (Figure 21A) and 81NE6 HMEC lines with elafin knockdown. Elafin-knockdown and control cells were cultured in growth factor-deprived medium, and cell number was assessed at the indicated time points (Figure 21B). Both 76NE6 and 81NE6 elafin-knockdown cell lines exhibited modest but significant growth factor-independent proliferation, with doubling times between 66 and 81 hours, while control cells exhibited complete growth cessation after only 24 hours in growth factor-depleted medium (Figure 21C).

To verify the specificity of elafin knockdown and examine the importance of elafin-mediated protease inhibition in this system, we complemented 76NE6 elafin-knockdown cells with wild-type elafin (sensitive to shRNA downregulation), shRNA-resistant-elafin, and shRNA-resistant-M25G-elafin (the M25G mutation inactivates the protease inhibitor domain) (636) (Figures 21D). Elafin-knockdown 76NE6 cells demonstrated an approximate doubling in cell number compared to controls following 144 hours of growth factor deprivation (Figure 21E). Complementation of elafin-knockdown 76NE6 cells with wild-type elafin, but not M25G-elafin, reduced cell number to the level of controls (Figure 21E).

These experiments revealed a critical role of elafin-mediated protease inhibition in the maintenance of G0 arrest in long-term growth factor-deprived HMECs.


A
76NE6
M.W.
Elafin
Actin
18
50
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Parental
Empty
Control
shElafin 1
shElafin 2
B
-24
0
24…72…120…168…240 hrs
Plate HMECs
Remove Growth Factors
Count Cells
C
76NE6
81NE6
Parental
Control
shElafin1
shElafin2
Cell Number (x10⁵)
Time (hours)
D
76NE6
M.W.
Elafin
Actin
18
50
0 48 96 0 48 96 0 48 96 0 48 96 hrs
Control
shElafin1 +wt
shElafin1 +R1
shElafin1 +R1M25G
E
76NE6
GF Deprivation 144-hours
Cell Number (x10⁵)
p<0.03
p<0.04
Control
shElafin1 + wt
shElafin2 + wt
shElafin1 + R1
shElafin2 + R2
S1 + R1 M25G
S2 + R2 M25G

Figure 21: Elafin Knockdown HMECs Circumvent Quiescence and Proliferate in a Growth Factors Independent Manner.

A) Western blot of elafin expression in parental, empty vector, non-targeting shRNA control, and elafin knockdown (shElafin 1 and shElafin 2) 76NE6 cells cultured with GF (0 hours) and without GFs for 48 and 96 hours. Actin, loading control. (B) Schematic of experimental design. 76NE6 cell lines from A were plated at an initial density of 2000 cells/well in 24-well plates and counted at the indicated times after growth factor removal. (C) Cell counts over time as described in B. Asterisks denote significant differences ($p<0.05$) between control and elafin-knockdown groups. (D) 76NE6 cells expressing elafin shRNA (shRNA1 [S1] and shRNA2 [S2]) were stably transduced with wild-type elafin (wt), shRNA-resistant elafin (R1, shRNA1-resistant elafin; R2, shRNA2-resistant elafin), or shRNA-resistant elafin with a M25G mutation (results for shRNA1 shown). Cell lysates were subjected to western blot analysis for elafin. Actin, loading control. (E) 76NE6 cells as described in D were cultured in the absence of GFs for 144 hours and counted.

Elafin Knockdown 76NE6 are Sensitive to the Growth Promoting Effect of NE.

Activated TAN contribute the majority of NE in the tumor microenvironment (542). Therefore, we assessed the ability of exogenous NE to induce the proliferation of HMECs. Elafin-knockdown and control 76NE6 cells were growth factor deprived for 24 hours followed by the addition of NE purified from human sputum directly to the media. Cell density was measured using the MTT assay 48 hours after addition of NE (72 hours after the removal of growth factors) (Figure 22A). Compared to controls, elafin-knockdown 76NE6 cells stimulated with NE at concentrations of 1-8 nM demonstrate a statistically significant increase in cell number (Figure 22B).

The ability of NE to induce proliferation was dose dependent. Elafin-deficient 76NE6 cells were sensitive to the growth-promoting effect of 2 nM NE, whereas elafin-expressing controls were not (Figure 22C). Complementation of elafin-knockdown 76NE6 cells with shRNA-resistant-elafin, but not shRNA-resistant-M25G-elafin, attenuated proliferation induced by 2 nM NE (Figure 22C). No differences were observed in this experiment between groups not treated with NE because cell number was assessed following only 72 hours of growth factor deprivation. [Significant differences in cell number were not seen in elafin-knockdown HMECs until 120 hours of growth factor deprivations (Figure 22C).] Pharmacological inhibitors of NE activity sivelestat and GW311616 inhibited NE-induced proliferation under all tested conditions (Figure 23, A and B); further evidencing that the mitogenic-effect of NE is dependent on NE-activity and not contaminates in the NE preparation.

These findings provided evidence that the growth-promoting effect of NE is dependent on its protease activity.

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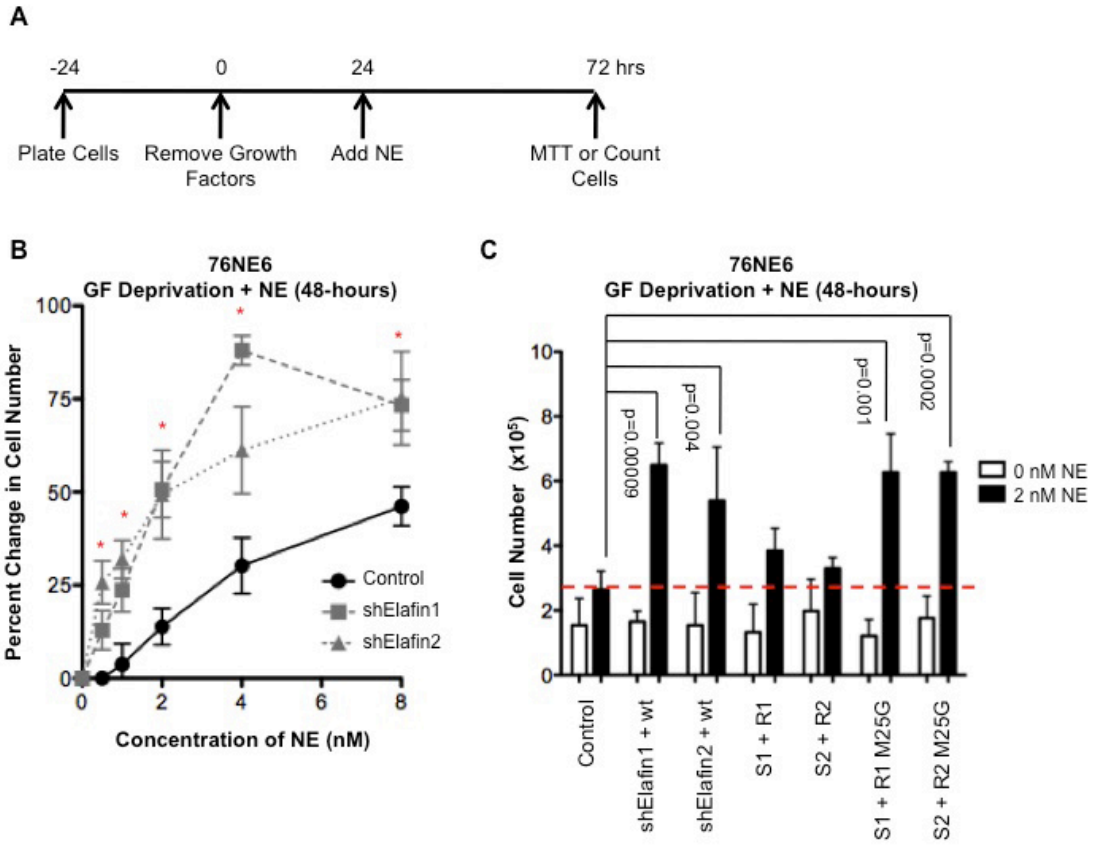


Figure 22: Elafin Knockdown 76NE6 are Sensitive to the Growth Promoting Effect of Exogenous NE.

(A) Schematic of experimental design; elafin knockdown and complemented 76NE6 cells described in figure 21 were growth factor deprived in DFCI-3 media for 24 hours, followed by addition of exogenous NE (purified from human sputum) in DFCI-3 media. Cell number was determined 48 hours after the addition of NE by either MTT assay or counting cells using a hemocytometer. (B) Elafin knockdown and control 76NE6 cells, percent change in cell density in response to 0, 2, 4, 6, and 8 nM NE, measured by MTT as described in A. (C) Control, elafin knockdown, and elafin complemented 76NE6 cells, cell number following stimulation with 2nM NE as described in A.

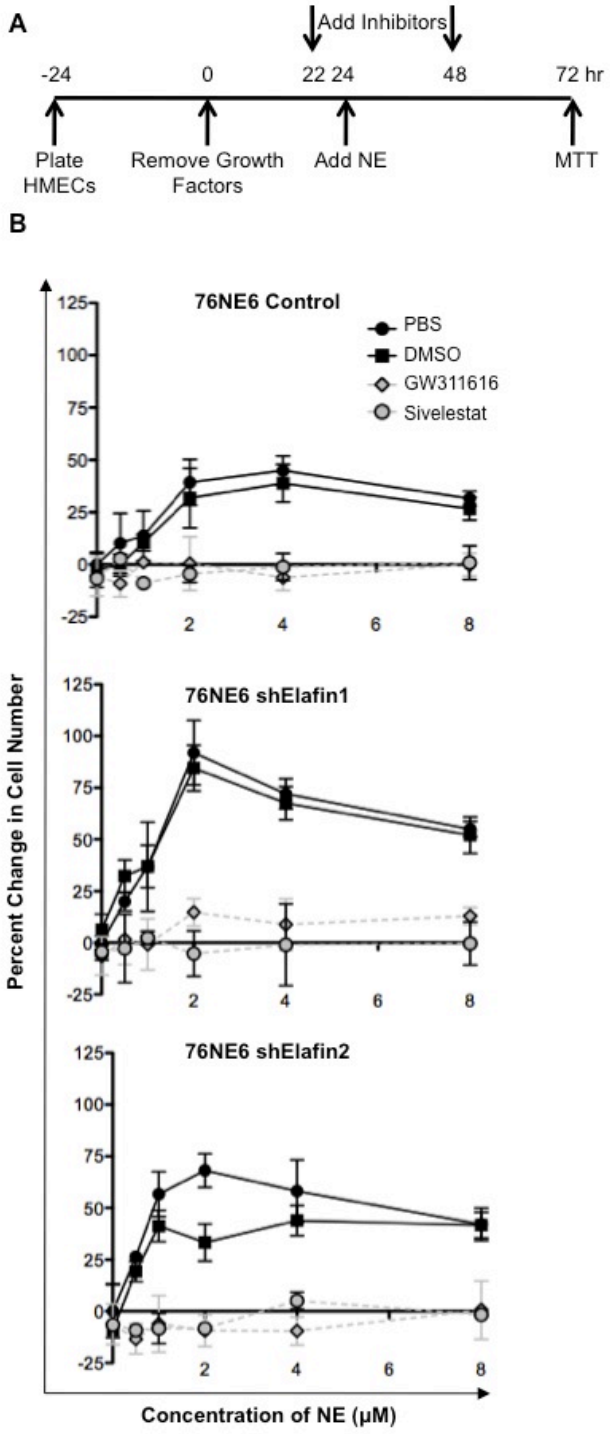


Figure 23: The Growth Promoting Effect of Exogenous NE is Dependant on its Proteolytic Activity

(A) Schematic of experimental design; elafin knockdown and control 76NE6 cells were growth factor deprived for 24 hours, followed by addition of 0, 0.5, 1, 2, 4, 6, and 8 nM NE for 48 hours. NE inhibitors GW311616 (20μM) and Sivelestat (100μM) were added two-hours prior and 24 hours post NE addition. (B) 76NE6 control and shRNA cell lines were treated with NE in the presence of PBS, DMSO, GW311616, or Sivelestat as described in A.

NE Induces the Deregulated Proliferation of Growth Factor Deprived HMECs Through TLR4-Dependent activation of ERK Signaling.

We performed microarray analysis to identify the molecular alterations underlying deregulated proliferation in growth factor-deprived elafin-knockdown HMECs. Genes differentially expressed between elafin-knockdown and control 76NE6 cells were identified following 0, 48, and 168 hours of growth factor deprivation (Table 6). Upregulation of the immediate early response gene, EGR1, at the 48-hour time point was of particular interest given the previously identified role of EGR1 in cell cycle re-entry of G0-arrested HMECs (714, 715). We validated the upregulation of EGR1 in 76NE6 elafin-knockdown cells by qPCR (Figure 24A). Complementation with shRNA-resistant elafin diminished EGR1 mRNA levels (Figure 24A). The ERK signaling pathway is known to control EGR1 transcription (715). In elafin-knockdown 76NE6 cells, the MEK inhibitor U0126 (10 μM) abrogated EGR1 upregulation (Figure S24, B and C) and inhibited proliferation (Figure S19D) following prolonged growth factor deprivation.

We chose to further explore the role of ERK signaling in NE-induced proliferation. 76NE6 cells were growth factor-deprived for 24 hours and then stimulated with 10 nM NE. The addition of NE resulted in activation of ERK phosphorylation that peaked within 15 to 30 minutes (Figure 25A). Upregulation of ERK target genes EGR1 and FOS was observed 3 hours after the addition of NE to growth-arrested 76NE6 cells (Figure 25B). Addition of U0126 (Figure 25C) to growth factor-deprived elafin-knockdown 76NE6 cells prevented proliferation induction by 2 nM NE (Figure 25D). Sivelestat, a specific NE inhibitor, also prevented proliferation induction by NE (Figure 25D). Given the rapidity of ERK activation following the addition of NE, we hypothesized that an extracellular receptor mediates the mitogenic effect of NE on HMECs. TLR4 (590), PAR2 (521), and EGFR (681) have all been implicated in NE-induced ERK activation. Knockdown of TLR4 reproducibly abrogated ERK activation in growth factor-deprived 76NE6 and 81NE6 cells (Figure 25E) following the addition of 10 nM NE and attenuated NE-induced proliferation (Figure 25F).

Both U0126 and MEK1 siRNA effectively inhibited ERK phosphorylation following the addition of NE to growth-arrested 76NE6 cells (Figure 26A) and consistently attenuated NE-induced proliferation (Figure 26, B and C). We transduced

76NE6 HMECs with shRNA specific to PAR2 and EGFR (shown is comparison to TLR4 knockdown). PAR2 was not detected in HMECs (data not shown) and was omitted from this analysis (Figure 27A). EGFR-knockdown HMECs demonstrated reduced ERK phosphorylation upon stimulation with 10 nM NE suggesting a role for EGFR in NE-induced ERK activation (Figure 27B). A role for EGR-1 was previously shown in EGF-induced cell cycle re-entry (716). However, knockdown of EGR-1 in 76NE6 cells (Figure 28A) was insufficient to block proliferation induced by NE, EGF, or serum-containing media (Figure 28B).

These findings suggested that the mitogenic activity of NE in quiescent HMECs is dependent on TLR4-induced ERK activation.

With Growth Factors		
Gene	Fold Change	p-value
PI3	-4.6	1.53E-07
SERPINB2	-1.6	1.40E-06
PAPPA	-1.5	3.60E-04
IGFBP3	-1.6	3.62E-04
Growth Factor Deprived 48 hours		
Gene	Fold Change	p-value
KRT13	1.7	1.00E-05
PI3	-2.8	1.20E-05
MMP10	-2.3	1.43E-05
VSNL1	1.6	1.03E-04
IGFL1	1.8	1.30E-04
HIST2H2AC	-1.5	1.44E-04
PMEPA1	-1.6	1.63E-04
FAM83D	1.5	3.01E-04
PTTG1	1.5	3.11E-04
CLCF1	-1.6	3.25E-04
EGR1	2.2	3.35E-04
IFI27	-1.7	4.44E-04
LAMA3	-1.5	5.01E-04
HSD17B2	-1.8	5.24E-04
IGFBP3	-1.8	5.26E-04
SPNS2	-1.6	6.48E-04
LOC647993	-1.5	7.45E-04
CLDN11	1.6	7.97E-04
Growth Factor Deprived 168 hours		
Gene	Fold Change	p-value
MMP7	2.8	2.08E-05
IFI27	1.6	2.48E-05
CFB	1.9	3.78E-05
ATP6V1B1	2.6	4.84E-05
CXCR7	1.6	5.36E-05
LOC653499	1.8	5.40E-05
OLFML2A	1.6	7.58E-05
PDPN	1.7	8.28E-05
KLHDC8B	1.6	1.54E-04
LGALS3	1.6	2.50E-04
CSPG4	1.5	2.54E-04
GDF15	1.5	3.94E-04
LGALS7B	1.9	4.25E-04

Table 7: Microarray Analysis Identifies EGR1 Upregulation in Growth Factor Deprived Elafin Knockdown 76NE6 HMECs

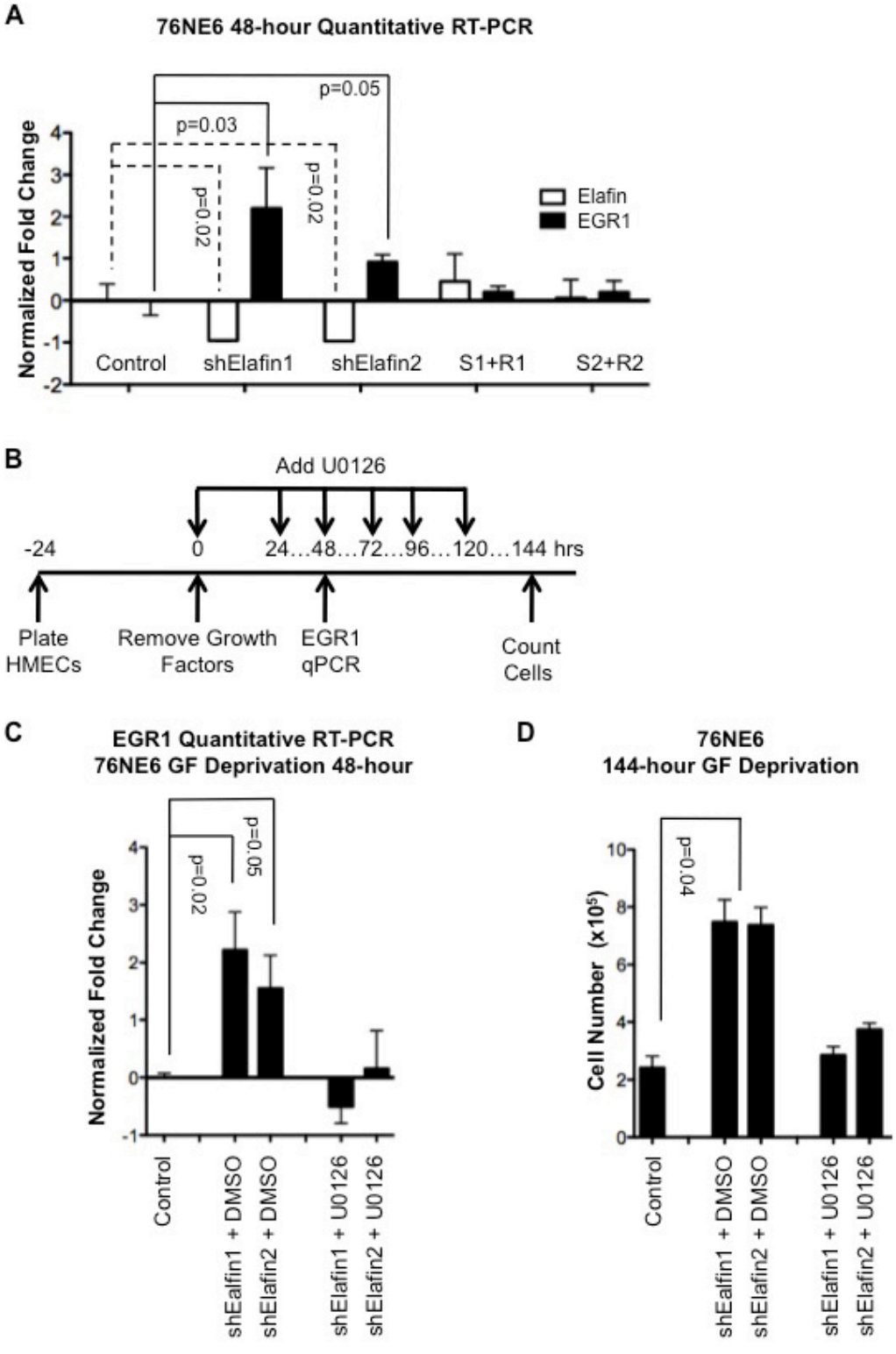

A
76NE6 48-hour Quantitative RT-PCR
p=0.05
p=0.03
p=0.02
p=0.02
Elafin
EGR1
Normalized Fold Change
Control
shElafin1
shElafin2
S1+R1
S2+R2
B
Add U0126
-24
0
24…48…72…96…120…144 hrs
Plate HMECs
Remove Growth Factors
EGR1 qPCR
Count Cells
C
EGR1 Quantitative RT-PCR
76NE6 GF Deprivation 48-hour
p=0.02
p=0.05
Normalized Fold Change
Control
shEalfin1 + DMSO
shElafin2 + DMSO
shElafin1 + U0126
shElafin2 + U0126
D
76NE6
144-hour GF Deprivation
p=0.04
Cell Number (x10^5)
Control
shEalfin1 + DMSO
shElafin2 + DMSO
shElafin1 + U0126
shElafin2 + U0126

Figure 24: The Growth Promoting Effect of Exogenous NE is Dependant on its Proteolytic Activity and Requires ERK Signaling

(A) Elafin knockdown and complemented 76NE6 cells described in figure 4D were cultured in growth factor depleted media for 48 hours and analyzed for elafin and EGR1 mRNA expression and normalized to control. (B) Schematic of experimental design; 76NE6 control, shElafin1, and shElafin2 were continuously cultured with 10 μM of the MEK inhibitor U0196 following growth factor removal. (C) Quantitative RT-PCR of EGR1 expression in U0196/DMSO treated elafin knockdown 76NE6 48 hours post growth factor removal, normalized as in B. (D) Cell number of U0196/DMSO treated elafin knockdown 76NE6 cells 144 hours post growth factor removal.

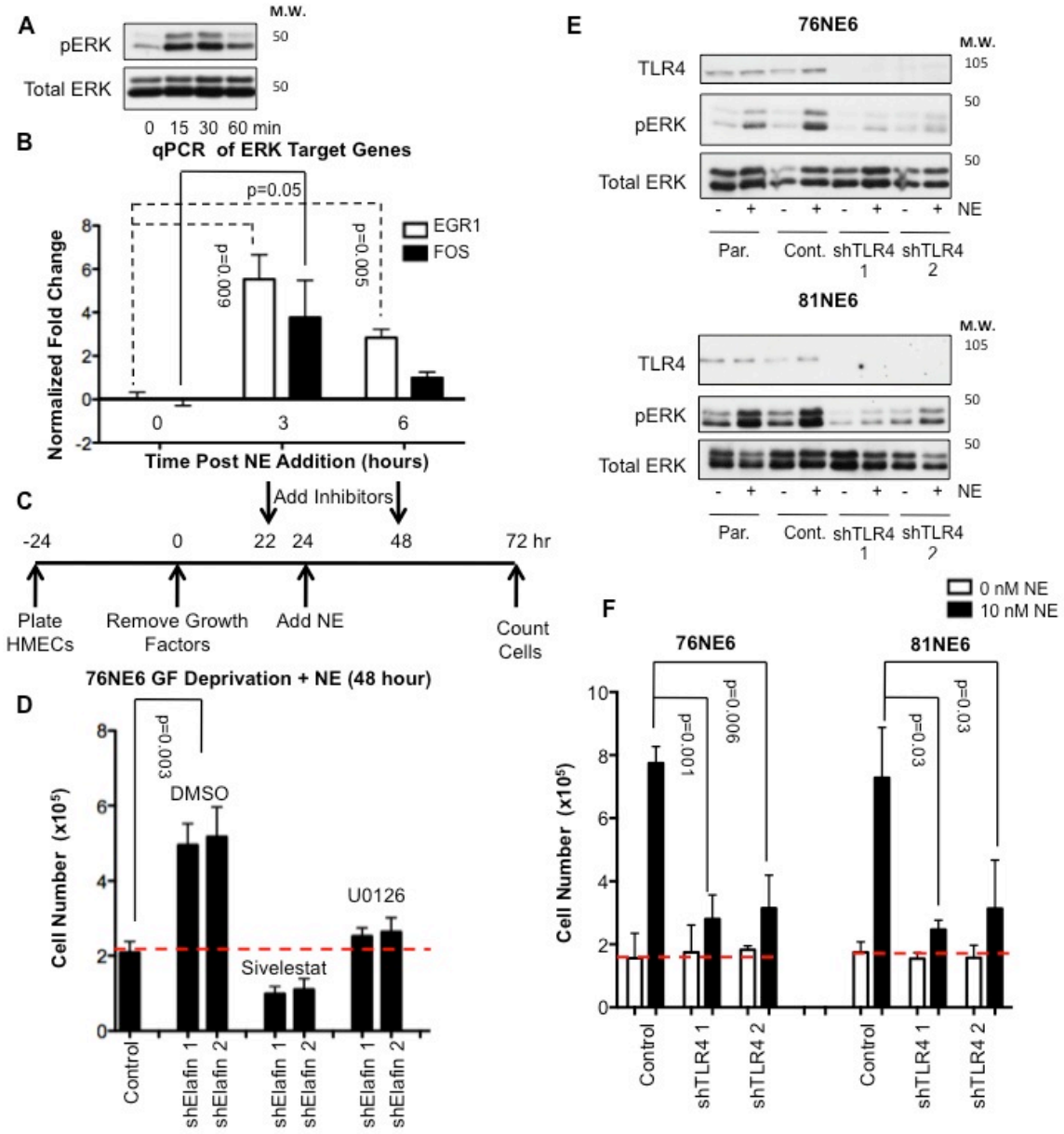

A
M.W.
pERK
Total ERK
50
50
0 15 30 60 min
B
qPCR of ERK Target Genes
p=0.05
p=0.009
p=0.005
EGR1
FOS
Normalized Fold Change
Time Post NE Addition (hours)
C
Add Inhibitors
-24
0
22
24
48
72 hr
Plate HMECs
Remove Growth Factors
Add NE
Count Cells
D
76NE6 GF Deprivation + NE (48 hour)
p=0.003
DMSO
U0126
Sivelestat
Cell Number (x10⁵)
Control
shElafin 1
shElafin 2
E
76NE6
TLR4
pERK
Total ERK
M.W.
105
50
50
NE
Par.
Cont.
shTLR4 1
shTLR4 2
81NE6
F
0 nM NE
10 nM NE
76NE6
81NE6
p=0.001
p=0.006
p=0.03
p=0.03
Cell Number (x10⁵)
Control
shTLR4 1
shTLR4 2

Figure 25: NE Induces the Deregulated Proliferation of Growth Factor Deprived HMECs Through the TLR4/EGFR-Dependant Activation of ERK Signaling

(A) Western blot analysis of pERK and total ERK in parental 76NE6 cells growth factor (GF) deprived for 24 hours and then stimulated with 10 nM NE. (B) qPCR analysis of EGR1 and FOS 3 and 6 hours after addition of NE. Values are represented relative to control (-GFs 24 hr). (C) Schematic of experimental design. Elafin-knockdown 76NE6 cells were GF deprived for 24 hours, and stimulated with 2 nM NE for an additional 48 hours. Two hours before and 24 hours after the addition of NE, DMSO, sivelestat (100 μM), or U0126 (10 μM) was added. (D) Control and elafin-knockdown 76NE6 cells described in C were counted. (E) Western blot analysis of TLR4, pERK, and total ERK expression in 76NE6 and 81NE6 TLR4-knockdown cells GF deprived for 24 hours followed by stimulation with 10 nM NE. Actin, loading control. Par., parental. Cont., control. (F) 76NE6 and 81NE6 TLR4-knockdown cells were counted 48 hours after the addition of 10 nM NE

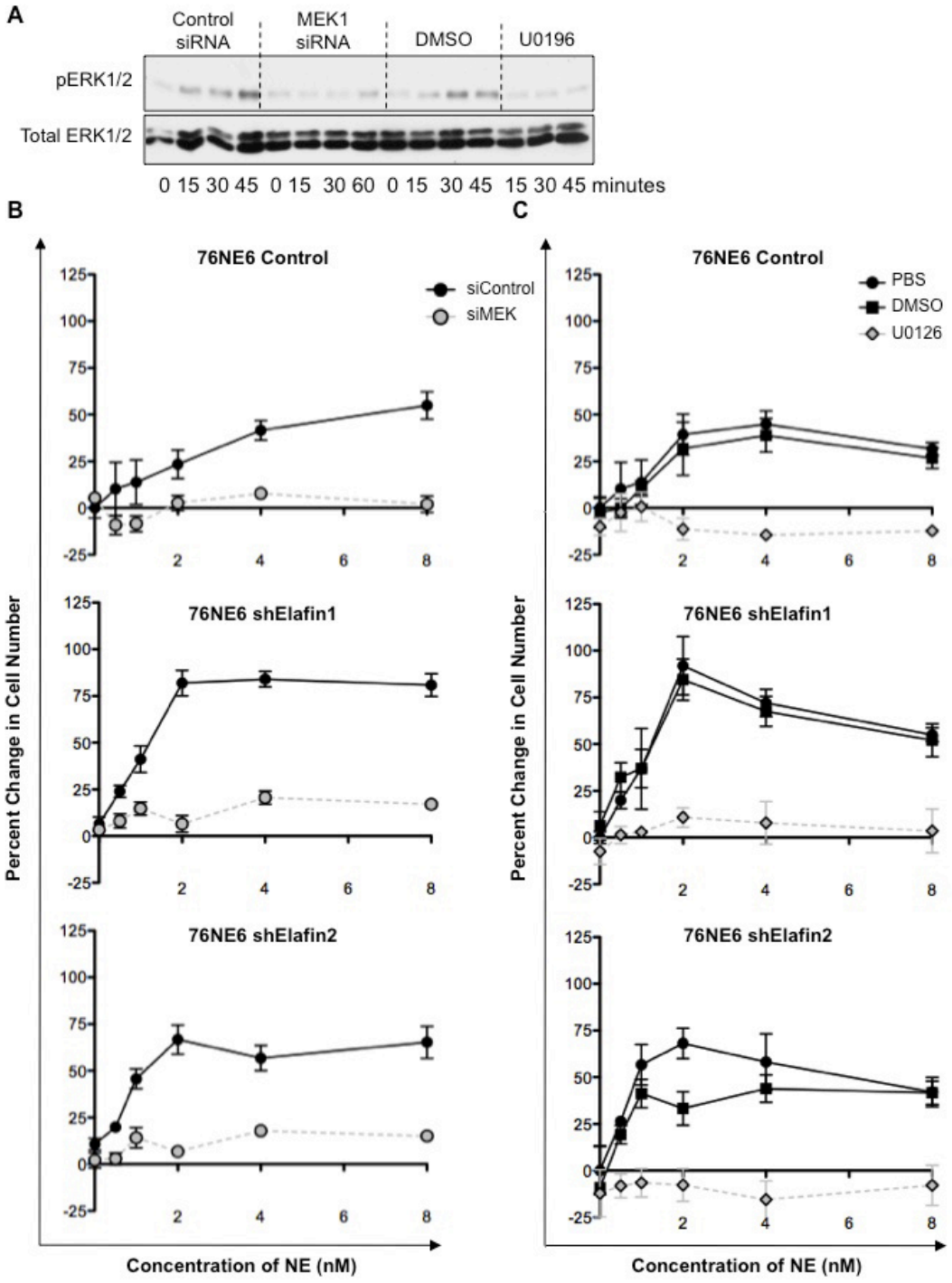

A
Control siRNA
MEK1 siRNA
DMSO
U0196
pERK1/2
Total ERK1/2
0 15 30 45 0 15 30 60 0 15 30 45 15 30 45 minutes
B
76NE6 Control
siControl
siMEK
76NE6 shElafin1
76NE6 shElafin2
Percent Change in Cell Number
Concentration of NE (nM)
C
76NE6 Control
PBS
DMSO
U0126
76NE6 shElafin1
76NE6 shElafin2
Percent Change in Cell Number
Concentration of NE (nM)

Figure 26: **The Growth Promoting Effect of Exogenous NE is Dependent on its Proteolytic Activity and Requires ERK Signaling**

(A) 76NE6 cells were transfected with MEK1 or control siRNA, growth factor deprived for 24 hours, and stimulated with NE (10 nM). Alternatively, 76NE6 cells were growth factor deprived for 24 hours and treated with DMSO or the MEK inhibitor, U0126 (10 μM), two-hours prior to NE addition. Cells were harvested 15, 30, and 45 minutes after NE addition and subjected to western blot analysis for phosphorylated (Thr202/Tyr204)- and total-ERK expression. (B) Elafin knockdown and control 76NE6 cells were prepared as above with MEK/control siRNA and stimulated with 0, 0.5, 1, 2, 4, and 8 nM NE for 48 hours, the percent change in cell number was calculated by MTT assay. (C) 76NE6 cells were examined as in B except for the use of U0126/DMSO/PBS (administered 2 hours prior and 24 hours post NE addition) instead of siRNA.

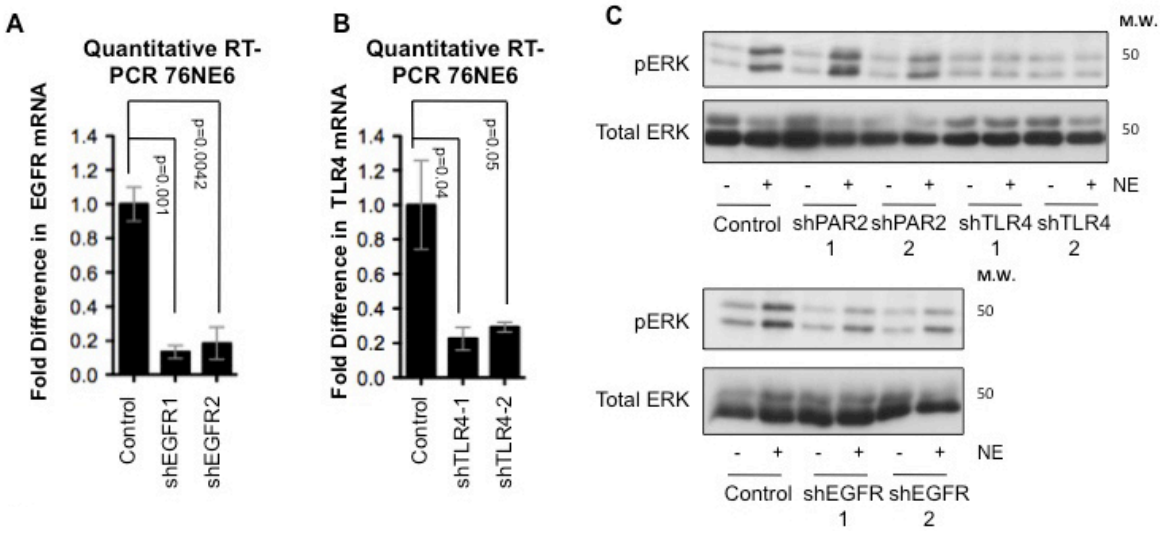


Figure 27: Knockdown of EGFR and TLR4

(A-D) 76NE6 cells were stably infected with pGIPZ lentiviral vectors containing control or two unique shRNAs targeting EGFR or TLR4. Knockdown of EGFR (A) and TLR4 (B) was confirmed by qPCR analysis. Values were normalized to GAPDH and represented as a ratio to the control shRNA level. PAR2 shRNA was also included; however, we were unable to detect PAR2 expression in these cells by qPCR. (C) Western blot analysis of phosphorylated (Thr202/Tyr204) and total ERK expression in 24-hour growth factor-deprived 76NE6 cells stably transduced with control shRNA or shRNA targeting PAR2, TLR4, and EGFR following stimulation with 10 nM NE or vehicle alone.

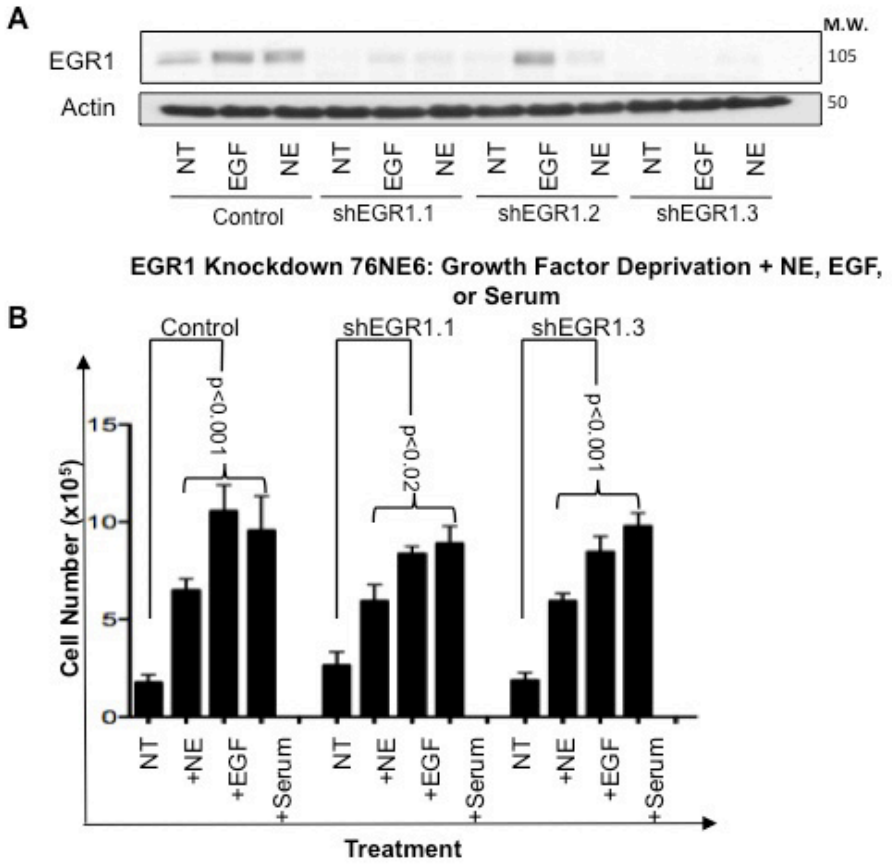


Figure 28: Knockdown of EGR-1 Fails to Prevent Cell Cycle Reentry Following Addition with NE, EGF, or Serum Containing Media

(A,B) 76NE6 cells were stably infected with pGIPZ lentiviral vectors containing control or three unique shRNAs targeting EGR1. The cells were growth factor deprived and then stimulated with 10 nM NE, 1 nM EGF, 10% serum containing media, or not-treated (NT). (A) Three hours after treatment the cells were harvested and lysates were subjected to western blot analysis. Both EGF and NE increased EGR1 levels compared to NT. EGR1 shRNA1 and shRNA3 abolished EGR1 upregulation. (B) Cells were counted 48 hours after treatment. NE, EGF, and serum containing media significantly increased cell number regardless of EGR1 levels.

DISCUSSION

Immunohistochemical analysis reveals that elafin is highly expressed in the normal epithelium of the breast and fallopian tube, but is switched off as breast and ovarian tumors achieve an invasive phenotype. HMECs served as a model system to understand the role of elafin in the normal mammary epithelium and to discover the rationale behind elafin downregulation during tumorigenesis.

A model based on the *in vitro* analysis performed in chapter two is presented in Figure 29. In this model, elafin is expressed by the normal quiescent epithelium under the transcriptional control of C/EBPβ, as a shield against neutrophil secreted NE activity (Figure 29A). During tumorigenesis elafin is downregulated due to deregulation of C/EBPβ, eliminating a critical counterbalance against the mitogenic effect of NE. We found that deregulated NE induces proliferation through TLR4-dependant activation of the ERK signaling pathway (Figure 29B). These results suggest a role for deregulated NE in promoting ERK-signaling and tumor growth.

Several published studies have observed elafin downregulation in invasive tumors and tumor-derived cell lines (21, 23, 24, 26). However, contradictory studies examining elafin expression in ovarian cancer and glioblastoma conclude that elafin overexpression is a marker of poor prognosis (717, 718). In an experimental glioblastoma model of anti-angiogenesis therapy, elafin upregulation was observed by microarray analysis along with CHI3L1, CHI3L2, and IL-1β in avascular when compared to vascularized tumors. In glioblastoma patient samples, elafin mRNA expression was associated with higher tumor grade and poor overall patieint survival. IHC examination of elafin is a small cohort of glioblastoma cases found elafin expression in cells adjacent to necrotic regions of the tumor (718).

The authors of the ovarian cancer study used a previously unpublished polyclonal antibody for immunohistochemical detection of elafin and were unable to detect elafin expression in normal ovarian epithelial cells (717). Aside from methodological discrepancies, the critical advantage of the immunohistochemical analysis presented here is the use of a scoring system based on elafin expression in normal tissue. Using this system, elafin overexpression can be defined as expression greater than that in the normal epithelium. Elafin overexpression, based on this criteria,

was observed in only 8% of breast tumors and <2% of ovarian tumors and had no prognostic significance. The Clauss et al. study scored elafin expression by the percentage of cells positive only. Tumors demonstrating elafin positivity in less than six-percent of cells were grouped into the low elafin group (77 of 134 tumor samples) and tumors demonstrating elafin positivity in greater than six-percent of cells were grouped into the high elafin group (57 of 134 tumor samples), suggesting that the anti-protease shield is still diminished in these tumors.

Alternative avenues of elafin transcription may differentiate the outcome of studies identifying elafin overexpression (717, 718) and downregulation (21-24, 26). Inducible elafin expression has been described in response to pro-inflammatory cytokines, especially IL-1β and TNF-α (646, 647), and is dependent NF-κB (717). C/EBP β sites (24) in the elafin promoter drive the expression of elafin in G0 HMECs (Figure 17). C/EBP β is essential to the constitutive expression of elafin in the normal epithelium.

In ovarian tumor cells, elafin expression was NF-κB-dependent (717). In glioblastoma, focal elafin expression was associated with necrotic tumor areas. Necrosis is associated with inflammatory cytokine production and NF-κB activation (718). Elafin immunohistochemical scoring based solely on invasive tumors therefore reveals an association with inflammation and necrosis, which is known to be associated with poor prognosis. Consistent with this observation, the relatively small cohort of breast tumors expressing elafin, at or above the level observed in the normal breast epithelium, correlates with histopathological markers of aggressive tumor behavior (Table 5). We hypothesize that inflammation and NF-κB activation results in elafin reactivation in this subset. Ranking the expression of genes that covariate with elafin in the TCGA breast cancer (85), Yau et al. 2010 (719), TCGA ovarian (720), and TCGA glioblastoma (721) cancer datasets finds that elafin expression is highly correlated with the expression of inflammatory genes, especially secreted factors, cytokines, chemokines, and proteases (Table 8). The role of elafin in tumors is unclear. Elafin has never been shown to have oncogenic functions in experimental models. Excessive inflammation and high levels of NE activity can lead to tissue destruction and apoptotic cell death (722-724). Elafin expression in aggressive tumors may be meaningless,

however elafin in these tumors may also function to maintain NE activity within a range that induces proliferation, but not cell death.

Elafin expression in G0 HMECs requires C/EBP β sites (24) in the elafin promoter (Figure 17). Alternative splicing results in the expression of C/EBP β as multiple isoforms, including a truncated transcriptional repressor. The accumulation of this dominant-negative C/EBP β isoform results in elafin downregulation in HMECs and tumor cells (24). We also found that Rb is required for elafin expression in HMECs (Figure 18). Physical interaction with Rb was previously shown to be critical for C/EBP β DNA-binding (725). In some cell types direct interaction between Rb and C/EBP β leads to enhanced transcriptional activity of C/EBP β target genes involved in quiescence and differentiation (726, 727). In fibroblasts, C/EBP β causes cell cycle exit in the presence of Rb, but accelerates proliferation in the absence of Rb (728). Deregulation of the Rb (85) and C/EBP β (24) pathways are a frequent event in human cancers and likely drives elafin downregulation during malignant progression.

Another explanation for the finding that elafin is overexpressed in a subset of breast and ovarian tumors is amplification of the PI3-gene. Amplification of the chromosomal region 20q13.2, where elafin resides, is frequently observed in ovarian cancer, breast, and other tumor types. Several candidate oncogenes have been identified in the region including NABC1, ZNF217, TPD54, ADRM1, and BTAK, however no consensus exists on the gene or genes driving amplification. 20q13.2 amplification is consistently associated with poor prognosis across a spectrum of tumor-types including breast and ovarian cancer (729-733). Elafin overexpression may be a readout on this amplification event.

The preponderate loss of elafin in invasive tumors compared to pre-invasive lesions suggests that elafin downregulation may play an important role in the transition to invasive carcinoma. Consistent with a tumor suppressive role for elafin in cancer, several published studies have observed cytotoxity following elafin re-expression in tumor cell lines and xenograft tumors (25, 26, 678).

In this study, we concentrated on the stoichiometric relationship (1:1 molar ratio) between elafin and NE. Under normal culture conditions HMECs do not express NE (25), however NE expression by epithelial cells has been reported in response to reactive oxygen species (734). Metabolic stress is a byproduct of growth factor

deprivation and may be driving inappropriate NE expression in HMECs. However, the low levels of proliferation observed in elafin KD HMECs following prolonged growth factor deprivation (Figure 21) likely reflects a low concentration of relevant protease activity in this system. NE expression has previously been characterized in tumor cell lines (617, 735), however in the tumor microenvironment the majority of NE is contributed by activated neutrophils (542, 616). To model this, we added purified NE to the media of G0 HMECs. The level of cell growth achieved in elafin KD HMECs 48-hours after addition of 2 nM NE (Figure 22) is comparable to the level observed following 144-hours of growth factor deprivation alone (Figure 21).

Several published studies have demonstrated increased proliferation following addition of exogenous NE (542, 676, 736). The concentrations of NE resulting in a physiological response *in vitro* vary between studies; proliferation was induced in airway smooth muscle cell by addition of 0.35-1.7 nM NE (736), in keratinocytes by addition of 0.1-33 nM NE (676), in lung fibroblasts by addition of 3.5-862 nM NE (681), and in lung cancer cells be addition of 20-80 nM NE (542). In the present study, the concentrations of NE utilized ranged from 1-10 nM to induce proliferation in HMECs, which is comparable to the concentrations used in most published studies. The specific activity of the purified NE, the intrinsic sensitivity of the cell lines, and the level of anti-proteases in the culture system are likely important factors in the concentration of NE required to generate a response. *In vivo* application of NE results in thickening of the epidermis and can be attenuated by addition of NE (676)

NE-induced ERK signaling and resultant proliferation is dependent on the TLR4. Direct cleavage of TLR4 by NE has been reported to induce its activation (737). In both leukocytes and epithelial cells, NE-induced TLR4 activation has an established role in the activation of the NF-κB pathway and downstream cytokines expression, such as IL-8, TNFα, and IL-6. TLR4 can also activate MAPK pathways including ERK signaling (590). We observed that knockdown of EGFR in HMECs partially inhibited NE-induced ERK activation (Figure 27). Stimulation of TLR4 by NE (560) or bacterial ligands (738) has previously been shown to result in activation of the EGFR, which is responsible for downstream ERK activation. In a non-small cell lung cancer (NSCLC) cell line, TLR4 ligands promote proliferation in an EGFR-dependent manner (739).

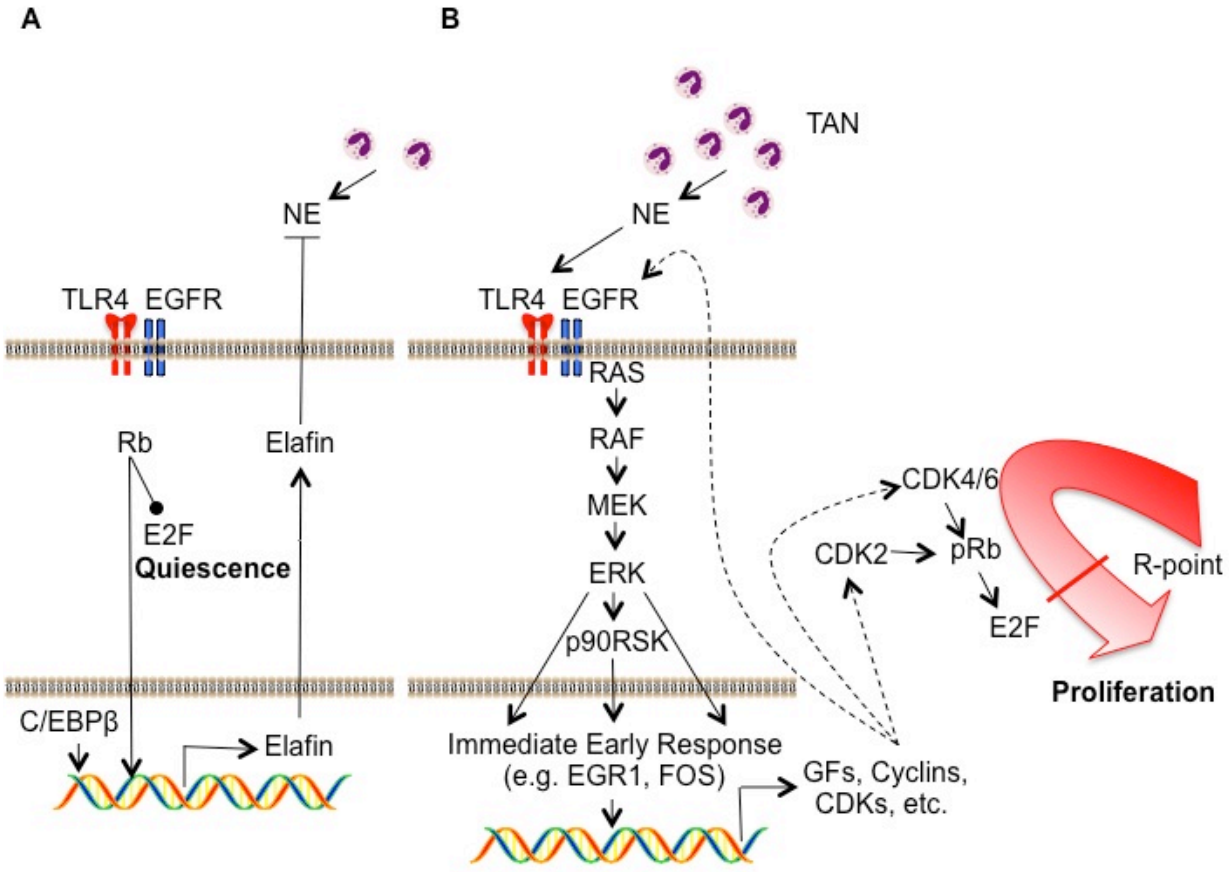


Figure 29: Proposed Model for NE-Elafin Equilibrium

(A) Elafin is expressed under the control of C/EBPβ and Rb in G0 HMECs. In this context, elafin blocks the mitogenic effects of secreted NE and maintains G0 arrest. (B) Loss of elafin expression and the accumulation of TAN-secreted NE in the tumor microenvironment lead to TLR4/EGFR-dependent activation of the ERK signaling pathway. The ERK pathway induces an immediate early gene response resulting in the transcription of growth factors, cell cycle regulatory factors and ultimately cell proliferation.

TCGA Breast Cancer			Yau et. al. Breast Cancer			TCGA Ovarian Cancer			TCGA Brain Cancer		
Rank	Gene	Corr.	Rank	Gene	Corr.	Rank	Gene	Corr.	Rank	Gene	Corr.
-	PI3	1.00	-	PI3	1.00	-	PI3	1.00	-	PI3	1.00
1	SLPI	0.67	1	SLPI	0.49	1	SLPI	0.54	1	CSF3	0.73
3	LY6D	0.60	8	CCL20	0.37	4	CXCL1	0.47	2	CCL7	0.73
4	CXCL1	0.60	10	CXCL1	0.36	5	S100A9	0.47	5	CCL20	0.66
11	CXCL3	0.55	11	S100A9	0.35	7	S100A8	0.46	6	SLPI	0.65
12	EGFR	0.55	13	S100A8	0.35	10	IL8	0.43	10	CXCL3	0.62
38	S100A8	0.50	17	MMP7	0.32	11	CCL20	0.43	18	IL8	0.59
44	CXCL2	0.50	24	MMP12	0.30	13	ICAM1	0.42	21	ICAM1	0.59
45	SERPINB5	0.50	26	CEBPB	0.30	14	TNF	0.42	25	CXCL2	0.58
49	SERPINB4	0.49	33	IL8	0.29	16	CEBPB	0.41	31	S100A9	0.55
56	CX3CL1	0.49	38	SERPINB5	0.28	19	S100A12	0.40	32	MMP7	0.55
60	SERPINB3	0.48	43	CX3CL1	0.28	20	CXCL5	0.39	36	S100A8	0.54
68	S100A9	0.48	47	SERPINB3	0.27	36	CXCL3	0.36	41	CCL2	0.54
70	CEBPB	0.47	61	ICAM1	0.26	39	CXCL2	0.36	43	IL6	0.54
78	S100A12	0.46	64	CXCL2	0.25	52	CXCL6	0.34	48	CXCL6	0.54
96	S100A2	0.45	71	CXCL5	0.25	54	MMP9	0.34	53	CXCL5	0.53
132	CXCL6	0.43	74	LY6D	0.25	57	S100A7	0.34	141	CEBPB	0.45
n=599; sig. p<0.0001			n=683; sig. p<0.0001			n=565; sig. p<0.0001			n=483; sig. p<0.0001		

TCGA Breast Cancer Luminal A			TCGA Breast Cancer Luminal B			TCGA Breast Cancer HER2+			TCGA Breast Cancer Basal-like		
Rank	Gene	Corr.	Rank	Gene	Corr.	Rank	Gene	Corr.	Rank	Gene	Corr.
-	PI3	1.00	-	PI3	1	-	PI3	1.00	-	PI3	1.00
5	CXCL3	0.64	4	SLPI	0.59	6	SLPI	0.7	3	SLPI	0.57
11	CXCL2	0.59	20	S100A8	0.43	16	S100A8	0.64	24	S100A8	0.46
43	SLPI	0.54	60	S100A9	0.38	14	CXCL2	0.64	41	S100A9	0.43
71	CXCL1	0.52	257	EGFR	0.29	17	S100A9	0.64	119	CXCL1	0.38
64	EGFR	0.52	371	CXCL3	0.27	59	CXCL1	0.56	339	CXCL3	0.32
1107	S100A9	0.29	398	CXCL1	0.26	81	CXCL3	0.53	308	EGFR	0.32
1644	CEBPB	0.25	541	CXCL2	0.24	90	CEBPB	0.52	373	CXCL2	0.31
1724	S100A8	0.24	1064	CEBPB	0.2				557	CEBPB	0.29
n=212; sig. p<0.0001			n=113; sig. p<0.03			n=49; sig. p<0.0001			n=86; sig. p<0.008		

Table 8: Inflammatory Gene Expression Covariates with the Expression of Elafin in Tumors.

Tumor gene expression data from the TCGA breast, ovarian, and gliobastoma projects and the Yau et al. 2010 breast tumor dataset was examined for correlation with elafin. All genes were compared to elafin (PI3) and ranked by Pearson product-moment correlation coefficient (Corr.). P values were calculated based on the t-distribution. The TCGA breast tumor database was broken down further by intrinsic subtype.

Chapter 3: The Neutrophil Elastase Inhibitor, Elafin, Triggers Rb-Mediated Growth Arrest and Caspase-Dependent Apoptosis in Breast Cancer

INTRODUCTION

Endogenous Serine Protease Inhibitors in Tumorigenesis

Maspin is a member of the serpin family of serine protease inhibitors. Similar to elafin, maspin expression was originally identified as highly expressed in HMECs, but downregulated in breast tumor derived cells (740, 741). Maspin downregulation has been observed in several tumor types including melanoma, breast, prostate, and gastric cancers (742-745). In experimental models, maspin demonstrates tumor suppressor properties, including reduction of tumor growth, angiogenesis, invasion, and metastasis (741, 746, 747). Intracellular maspin (esp. nuclear) is a marker of good prognosis in breast cancer (748, 749). Recent studies suggest that nuclear localization of maspin is essential to its tumor suppressive activity (750). Intracellular maspin induces apoptosis in breast cancer cells by associating with the mitochondrial membrane and facilitating the release of cytochrome c. Maspin also modulates the expression of anti-apoptotic BCL-2 family members and promotes caspase activation (747, 751-753).

An essential feature of serpin protease inhibition is the exposed reactive center loop (RCL). The RCL makes contact with the active site of the protease target. Upon formation of an acyl-enzyme intermediate the serpin undergoes a rapid stressed to relaxed conformational change, distorting the active site of the target protease, and disfavoring hydrolysis. Following this reaction the RCL of the serpin remain covalently bound to the target protease, irreversibly inhibiting protease activity. The RCL of maspin is short and contains several hydrophobic residues that make it incapable of interacting with and inhibiting serine proteases (754). Therefore, the tumor suppressive properties of maspin are not dependent on an ability to inhibit protease activity.

Plasminogen activator inhibitor 1 (PAI-1) is another serpin with a role in tumorigenesis. PAI-1 is an active inhibitor of urokinase-type plasminogen activator (uPA), which has an established role in tumor progression. PAI-1 was originally

hypothesized to have a tumor suppressive role in cancer by opposing the tumor/metastasis enhancing activities of uPA. Several early studies supported a tumor suppressive role for PAI-1, however in breast cancer patients the expression of both uPA and PAI-1 are prognosis of poor prognosis (755-758). Protease independent roles have since been described for PAI-1 in enhancing tumor growth, angiogenesis, and metastasis (759).

WAP-domain containing, secretory leukocyte protease inhibitor (SLPI) has also been shown to have context dependent roles in tumor growth and progression (760, 761). Overexpression of both wild-type or protease inhibitor-dead SLPI enhanced proliferation, colony formation, survival, and xenograft tumor formation following overexpression in the HEY-A8 ovarian cancer cell line (760). Overexpression of SLPI enhances the tumorigenicity and metastatic capacity in the 3LL-S (Lewis Lung Carcinoma) cell line in a subcutaneous xenograft model. Contrarty to the results reported in the ovarian cancer cell line HEY-A8, ithe pro-tumorigenic capacity of SLPI n the 3LL-S cell line was dependnat on its protease inhibitory domain. (762, 763). Tumor suppressive effects of SLPI overexpression have also been reported. In the F3II BALB/c murine mammary carcinoma cell line, SLPI overexpression severely limited the ability of these cells to form tumors in mice (764). Overexpression of SLPI in the metastatic cell line H-59 reduces the ability of these cells to elicit a pro-inflammatory response in the liver and to form metastases (765). In a particularly interesting study, SLPI overexpression was shown to enhance the proliferation and growth of colon xenograft tumors, but inhibit the growth of mammary xenograft tumors. In vitro SLPI induces apoptosis in breast tumor cells, but not colon tumor cells (761).

Endogenous serine protease inhibitors play important roles in tumor progression. Interestingly, many studies have identified protease-independent role for protease inhibitors in tumorigenesis and progression. Protease-independent function (673) have also been identified for elafin. There is no known role for elafin in tumorigenesis.

Hypothesis and Central Findings

Several published studies suggest that elafin expression is downregulated during tumorigenesis including in squamous cell carcinomas of the skin, head/neck, and

esophagus (21, 22, 677, 766). Data presented in chapter 2 of this dissertation clearly demonstrates that elafin is downregulated in breast and ovarian cancer patients. The downregulation of elafin during tumorigenesis suggests that elafin possesses tumor suppressive properties. The majority of breast tumor derived cell lines demonstrate transcriptional downregulation of elafin, when compared HMECs (23).

The central hypothesis of this chapter states that elafin has tumor suppressive activity in breast cancer. The observations presented here suggest that the Rb pathway governs the anti-tumor properties of elafin. In breast cancer cells with functional Rb, the expression of elafin triggered Rb-dependent cell cycle arrest. Elafin also exhibited suppressive activity in breast cancer cell lines lacking Rb, but this was associated with an induction of caspase-3 dependent. Normal mammary epithelial cells were not affected by elafin. Collectively, these results argue that elafin mediates tumor suppressive effects that are cytostatic or cytotoxic depending on Rb status. These findings suggest that elafin could be engineered as a therapeutic modality to treat breast cancer without toxicity to normal proliferating cells.

MATERIALS AND METHODS

The methodology for DNA content analysis, preparation of protein lysates, western blot analysis, growth curves, and MTT assay are detailed in chapter two, additional reagents and protocols are described here.

Antibodies

Primary antibodies used for western blot (WB) or immunoprecipitation (IP):

Antibody	Species	Clone	Company	Application	Notes
Rb	Mouse monoclonal	4H1	Cell Signaling Technology	WB	Detects only phosphorylated Rb
Phospho-Rb site Ser807/811	Rabbit Polyclonal		Cell Signaling Technology	WB	
PARP	Rabbit Polyclonal		Cell Signaling Technology	WB	
Caspase 3	Rabbit Polyclonal		Cell Signaling Technology	WB	
Cleaved Caspase 3 Asp 175	Rabbit Polyclonal		Cell Signaling Technology	WB	
CDK4	Rabbit Polyclonal		Santa Cruz Biotechnology	IP	

Cell Lines and Culture Conditions

All cell lines were maintained in a humidified tissue culture incubator at 37ºC and 6.5% CO_2. All tumor derived cell lines (MDA-MB-157, MDA-MB-231, MDA-MB-468, MCF-7, ZR75.1, and T47D) were obtained from ATCC. Between 18 and 36% of established cell lines are cross contaminated or incorrectly identified (767), therefore all cell lines utilized were authenticated by the short tandem repeat method. All cell lines were also tested for mycoplasma contamination using the MycoAlert kit (Lonza). MDA-MB-468 cells were maintained in DMEM (HyClone) supplemented with 10% fetal calf serum (Atlanta Biological), 0.01M HEPES, 2 mM L-glutamine, and 0.1 mg/mL of ciprofloxacin. The remaining tumor cell lines were maintained in α-MEM (HyClone)

supplemented in the same manner as DMEM with the addition of 0.1 mM non-essential amino acids and 1 mM sodium pyruvate.

The pan-caspase inhibitor zVad-fmk (calbiochem) was solubilized in DMSO at 50 mM and diluted in media at a concentration of 50μM. Cyclohexamide was dissolved in PBS at a concentration of 1 mg/mL, filtered, and diluted in media to a final concentration of 100 μg/mL.

Knockdown/Overexpression in Breast Cancer Cell Lines

MDA-MB-231 and MCF-7 cells stably expressing shRNA directed against Rb were a gift from Dr. E. Knudsen; these cells were maintained in α-MEM supplemented with 2 ug/mL puromycin (Sigma). MCF-7 cells with reconstituted caspase 3 were a gift from Dr. B. Fang; these cells were maintained in α-MEM supplemented with 0.1 mg/mL G418 (Mediatech). MCF-7 RBKD and MCF-7 shRNA control cell lines expressing caspase 3 or pcDNA3.1 empty vector were generated by transfection of the MCF-7 RBKD and MCF-7 shRNA control cell lines with 6 μg of pcDNA3.1/Casp3 (also from Dr. B. Fang) or 6 μg of pcDNA3.1 empty vector using Genejuice reagent (Novagen), according to manufactures instructions. Cells expressing these vectors were selected in α-MEM containing 0.5 mg/ml G418 (Mediatech) and 2 ug/mL puromycin (Sigma) for four weeks. Single-cell clones were selected and expanded in culture media supplemented with 0.1 mg/ml G418 and 2 ug/mL puromycin (Sigma) and screened by western blot analysis for the expression for Rb and caspase 3.

Production of Adenovirus and Infection of Target Cells

Elafin and Firefly Luciferase transgenes were expressed downstream of a cytomegalovirus (CMV) promoter in replication incompetent adenovirus type 5. Viruses were amplified in the packaging cell line AD-293 (Stratagene) and purified in a CsCl gradient by centrifugation at 176 000 g (Beckman ultracentrifuge) at 4^{o}C for 18 hours. Multiplicity of Infection (MOI) was calculated based on the number of plaque forming units (pfu) in AD-293 cells. For infections 2 x 10^{6} cells were plated on a 100mm plate, after 24 hours cells were washed with sterile PBS and treated with AD-elafin, AD-luciferase, or PBS in four mL of media. After two hours of infection the media was removed and replaced with fresh culture media. All tumor cell lines were optimally

infected at 1 MOI except for MDA-MB-231, which along with HMEC cell lines were infected at 2 MOIs. Transgene expression was determined by western blot analysis. Infection efficiency was monitored using an identically tittered adenovirus expressing cDNA corresponding to GFP, all cell lines were infected at greater than 90% efficiency.

BrdU incorporation

Cells were plated in a 100 mm^3 plate and treated 24 hours later with Ad-Elafin, Ad-Luc, or PBS. 48 hours post-treatment, cells were pulsed for 1 hour with 10 μM BrdU (Invitrogen), harvested, washed in PBS, and fixed in ice cold 70% ethanol for at least 24 hours. Following fixation, 2 x 10^6 cells were aliquoted from each condition and washed in wash buffer (PBS + 1% BSA). The DNA was slightly denatured in 2M HCL, the cells were rinsed in wash buffer, residual acid was quenched by addition of 0.1 M sodium borate, and the rinsed again in wash buffer. Cells were then incubated in the dark for 1 hour on a rocker in 50 uL of dilution buffer (PBS + 0.5% tween-20 + 1%BSA) containing a 1:10 dilution of FITC-conjugated anti-BrdU monoclonal antibody (BD Bioscience). Cells were washed in buffer to remove background and resuspended in 0.5 mL of propidium iodide (PI) (Molecular Probes) staining solution (10 ug/mL PI + 20ug/mL Ribonuclease A (Sigma) in wash buffer) for 30 minutes at RT. PI and FITC fluorescence was measured using a BD FACScalibur flow cytometer and analyzed using FloJo software.

TUNEL Assay

To assess the percentage of cells that had undergone apoptosis, cells were treated as indicated and harvested 72 hours post treatment. Both the floating and adherent cells (by trypsin) were collected and 2 x 10^6 cells were aliquoted per condition and washed in PBS. Cells were fixed on ice for 20 minutes in 2.5% (w/v) paraformaldhyde (Sigma) in PBS (pH 7.4) and post-fixed in ice cold 70% ethanol for at least 24 hours. The TUNEL reaction was performed using the APO-BrdU TUNEL Assay Kit (Invitrogen) according to manufactures instructions. The included Alexa-Fluor 488 conjugated anti-BrdU antibody was used in all cases except where GFP expression from stably transfected plasmids (RBKD and control cell line) interfered with detection of the Alexa-Fluor 488 dye; in this case Alexa-Fluor 647 conjugated Anti-BrdU antibody

(BD) was substituted.

Immunoprecipitation-Kinase Assay

For determination of CDK4 kinase activity, 1 µl/sample of polyclonal rabbit CDK4 antibody was added to 30 µl/sample of protein A sepharose beads (GE HealthCare) at a concentration of 0.1 g/mL in lysis buffer (0.5M tris, pH 7.5, 2.5M sodium chloride, 1% NP-40 (Igepal)) and 36 µl/sample of 10 mM DTT/lysis buffer/PPI and incubated overnight, at 4^{o}C on a rocker. The following day, 200 µg of protein samples were added to the mixture and incubated for one hour, at 4^{o}C with rotation. The beads are then washed in 500 µl of the lysis buffer three times and then washed an additional three times in 500 µl of kinase wash buffer (10mM $MgCl_2$, 1mM DTT, 0.1 mg/ml BSA, 25mM Tris HCL pH 7.5, 125mM NaCl). All liquid is then removed from the reaction and the beads are resuspended in the kinase reaction buffer: 10mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA, 25 mM Tris HCL pH 7.5, 125mM NaCl plus 60mM ATP, 5µ Ci of γ-^{32}P ATP and 1µg of GST-Rb 769 substrate (Santa Cruz). The reaction was allowed to proceed for 30 minutes at 37ºC and inactivated by addition of 15µl of sample buffer. The samples were resolved on a 10% SDS-PAGE gel, stained in Brilliant Blue (Sigma) for one hour and de-stained overnight. The gel is then dried onto filter paper, exposed to a phosphorimager screen, and analysis by a Typhoon scanner (Molecular Dynamics).

RESULTS

Exogenously Expressed Elafin Induces Apoptosis in HMECs Lacking Functional Rb.

In Chapter two, we demonstrate that actively proliferating HMECs in growth factor containing media do not express elafin. However, elafin is highly upregulated in growth factor deprived, G0-arrest HMECs (Figure 15). In the HMECs, elafin is required for maintenance of G0-arrest during long-term growth factor deprivation conditions (Figure 21). To determine if exogenously expressed elafin can affect growth of actively proliferating HMECs, elafin was overexpressed using an adenoviral vector. Adenoviral expression of luciferase was used as a control for viral infection. Mock transduced cells were treated with an equivalent volume of PBS.

Sub-confluent 76NE6, 76NF2V, and 76NE7 cells were treated with adenoviral elafin, adenoviral luciferase, or PBS. Lysates were subjected to western blot analysis to demonstrate expression of the elafin transgene in comparison to endogenous elafin expression in growth factor deprived 76NE6 cells (Figure 30A-compare lane C [control: 76NE6 –GFs 48 hr], to lane E for each cell line). Adenoviral expression of elafin in proliferating HMECs produces expression levels comparable to growth factor deprived 76NE6 cells (Figure 30A). The overexpression of elafin causes no statistically significant change in the doubling times of Rb-proficient cell lines 76NE6 (PBS: 16±1.1 hours, luciferase: 15±0.5 hours, and elafin 15±0.3 hours) and 76NF2V (PBS: 18±0.6 hours, luciferase: 18±0.6 hours, and elafin 20.3±0.5 hours), suggesting that elafin expression alone is insufficient to induce growth arrest. However, overexpression of elafin in 76NE7 cells, which are devoid of Rb, resulted in a significant decrease in cell number by 96 hours post-treatment (Figure 30B).

To determine if the decrease in cell number seen in 76NE7 cells (Figure 30B) is due to adenoviral elafin-induced cell cycle arrest or induction of apoptosis we analyzed BrdU incorporation (Figure 30C) and TUNEL staining (Figure 30D). There was no statistically significant change in BrdU incorporation following ectopic expression of elafin in any of the cell lines tested (76NE6, 76NF2V, or 76NE7) (Figure 30C). There was a significant increase in TUNEL positive cells in adenoviral elafin treated 76NE7

cells compared to adenoviral luciferase treated 76NE7 or adenoviral elafin treated 76NE6 or 76NF2V cells (Figure 30D). The TUNEL assay measures DNA fragmentation, a result of apoptotic cell death.

These results suggest that elafin induces apoptosis preferentially in Rb deficient HMECs (76NE7) compared to Rb-expressing HMECs (76NE6 and 76NF2V).

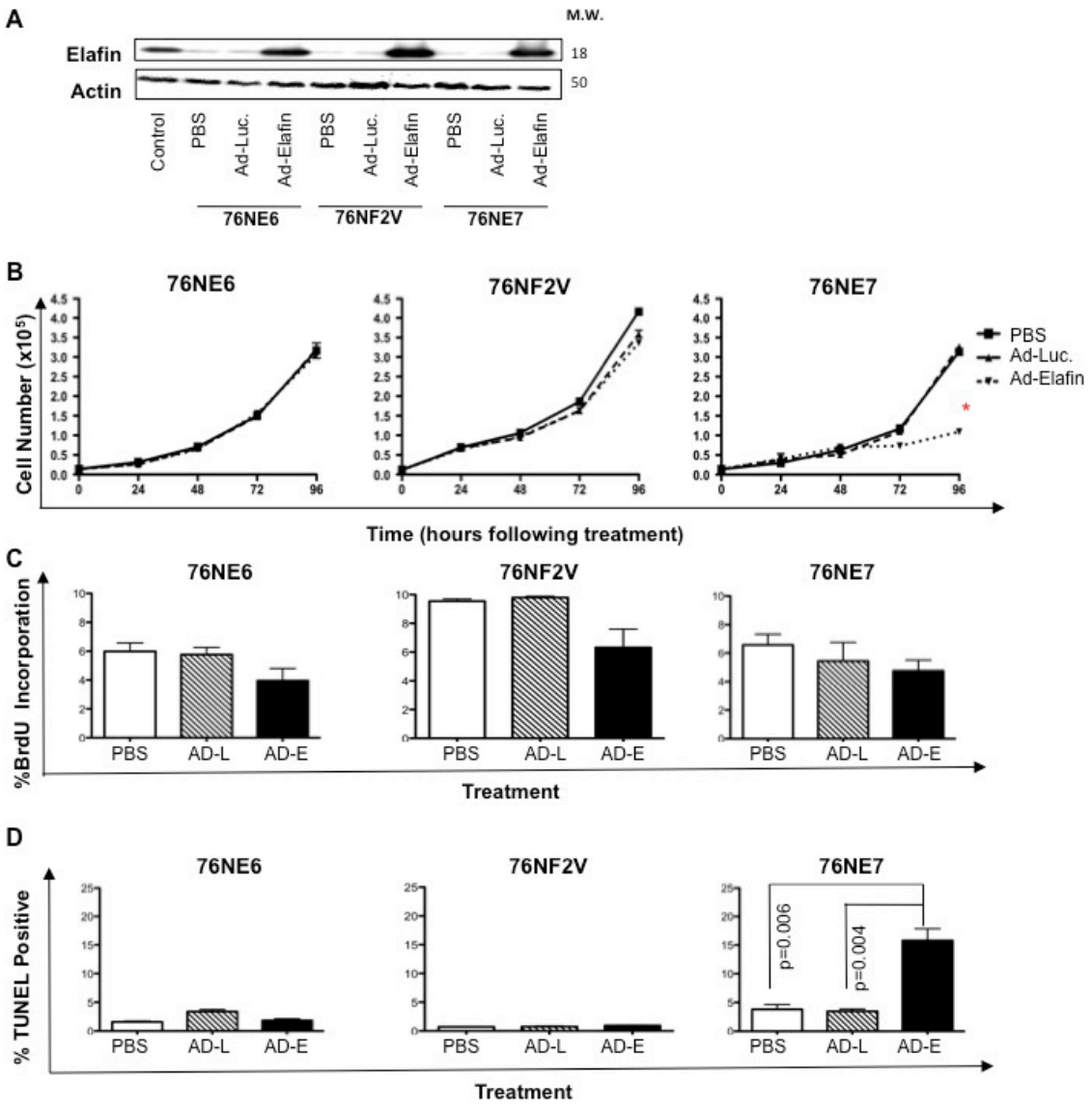


Figure 30: Exogenously Expressed Elafin Induces Apoptosis in HMECs Lacking Functional Rb.

(A-D) 76NE6, 76NF2V, and 76NE7 cells were treated with PBS, adenoviral luciferase (Ad-L), or adenoviral elafin (Ad-E). (A) Cells were harvested 48 hours post-treatment and subjected to western blot analysis for elafin expression. Actin, loading control. (B) Growth was monitored by trypan blue exclusion test every 24 hours for 96 hours. Asterisk indicates statistical significant. (C) Proliferation was assessed 48 hours post-treatment by measuring BrdU incorporation. (D) Apoptosis was assessed 72 hours post-treatment by TUNEL assay.

Expression of Elafin in Rb Negative Breast Cancer Cells Results in Apoptosis.

Based on the finding that elafin induces apoptosis in Rb deficient HMECs, we hypothesized that Rb deficient breast cancer cell lines are susceptibility to elafin-induced apoptosis. We examined elafin re-expression in three breast cancer cell lines with functional/wild-type Rb (MCF-7, ZR75.1, and T47D) and three cell lines deficient in Rb (MDA-MB-157, MDA-MB-468 and MDA-MB-436). All six cell lines were treated with adenoviral elafin, adenoviral luciferase, or PBS and were examined for growth kinetics, BrdU incorporation, and TUNEL positivity (Figure 31). Western blot analysis of adenoviral transduced cells reveals restoration of elafin expression in breast cancer cell lines comparable to control (76NE6 cells growth factor deprived for 48 hours), suggesting that elafin is being expressed at physiologically relevant levels (Figure 31A). Treatment of Rb positive breast cancer cell lines with adenoviral elafin resulted in a modest growth inhibition, whereas treatment of Rb negative cell lines with adenoviral elafin resulted in a precipitous decline in cell number (Figure 31B).

All three of the Rb expressing cell lines demonstrated a significant reduction in BrdU incorporation, while the Rb deficient cell lines demonstrated no significant change in BrdU incorporation (Figure 32A). Examination of TUNEL positivity fails to identify an increase in apoptotic cell death in Rb positive cell lines in response to adenoviral elafin. However, all three Rb negative cell lines underwent significant levels of apoptosis in response to elafin expression (Figure 32B). The percent TUNEL positivity ranged between 22-30% of all Rb negative cells treated with adenoviral elafin, compared to 1-5% of cells treated with adenoviral luciferase ($p<0.01$).

These results suggest that elafin overexpression results in growth inhibition in Rb positive cells and apoptosis in Rb negative breast cancer cells.

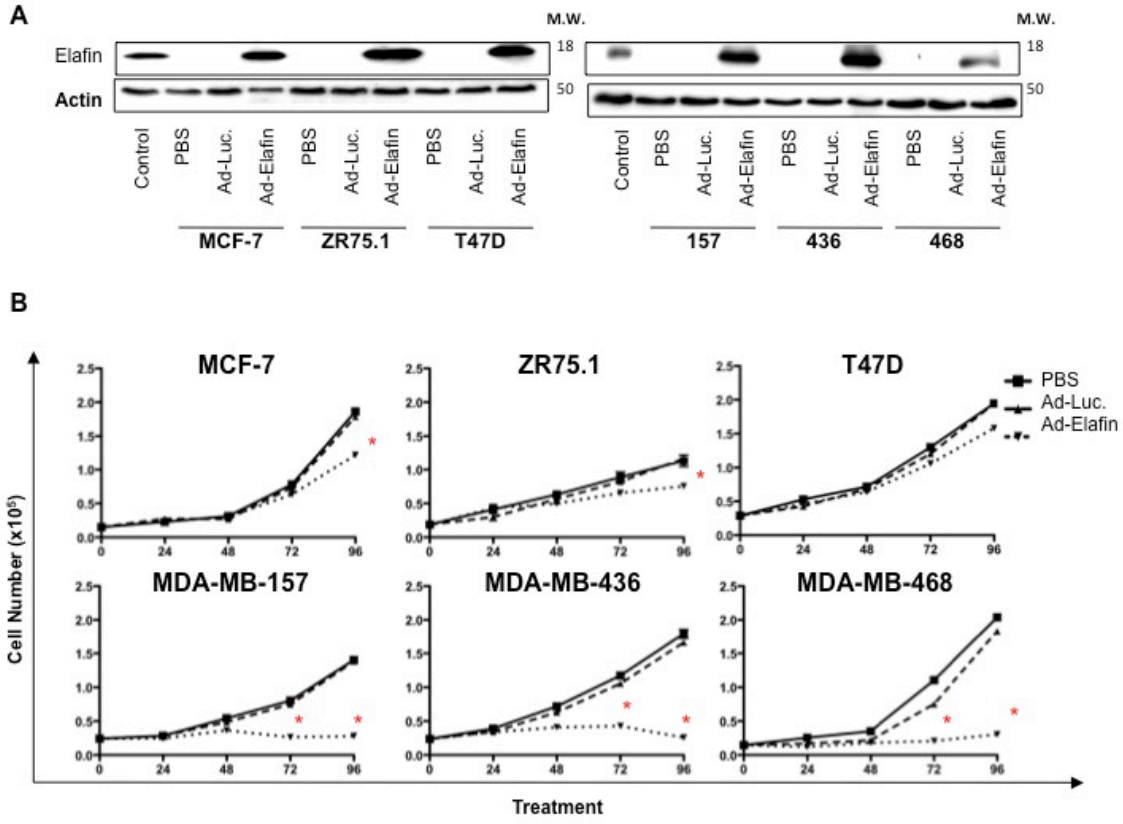


Figure 31: Expression of Elafin in Rb Negative Breast Cancer Cells Results in Apoptosis.

(A,B) MCF-7, ZR75.1, T47D, MDA-MB-157, MDA-MB-436, and MDA-MB-468 cells were treated with PBS, adenoviral luciferase, or adenoviral elafin. (A) Cells were harvested 48 hours post-treatment and subjected to western blot analysis for elafin expression. Actin, loading control. (B) Growth was monitored by trypan blue exclusion test every 24 hours for 96 hours. Asterisks indicate a statistically significant change in cell number, elafin treated compared to controls.

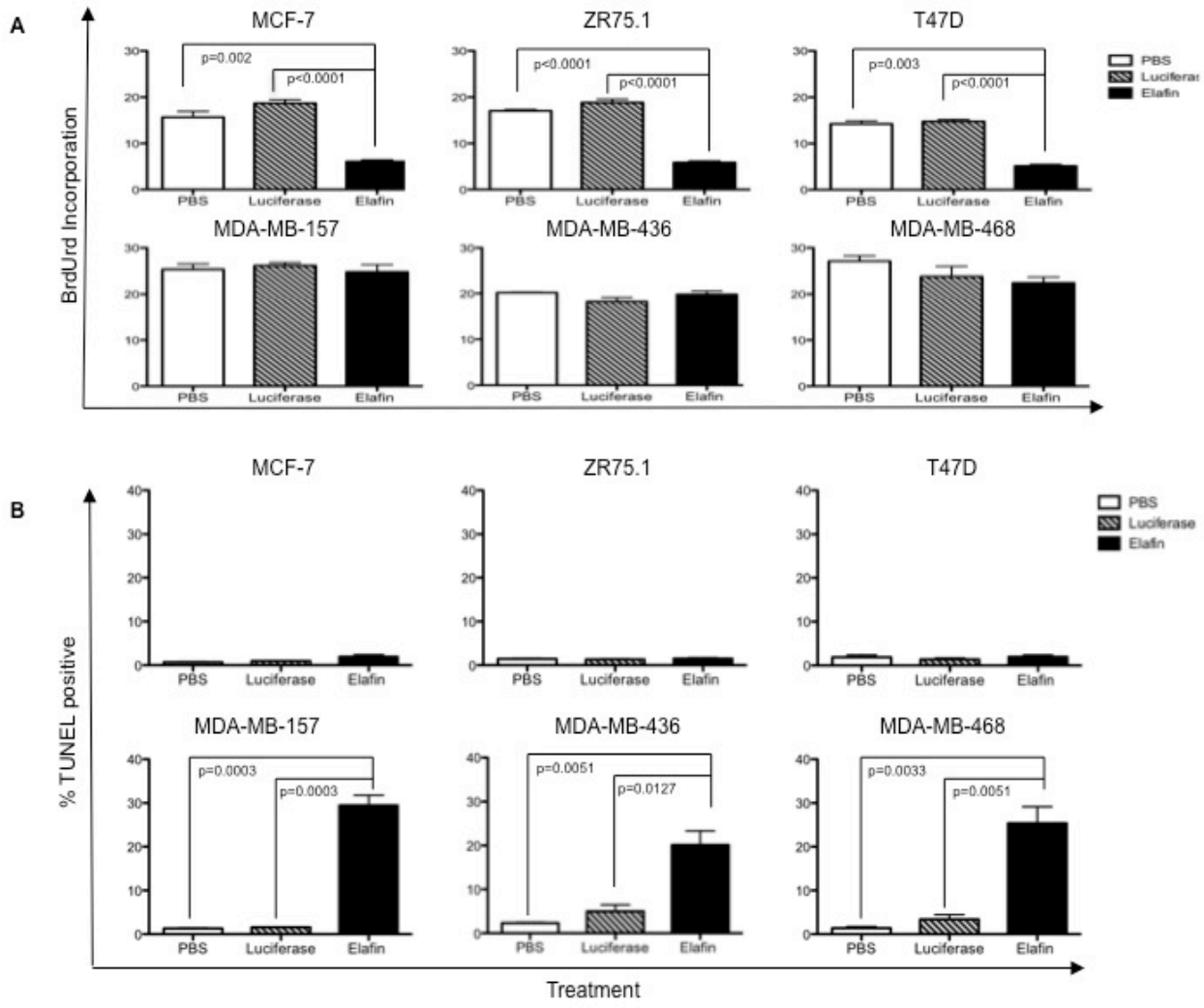
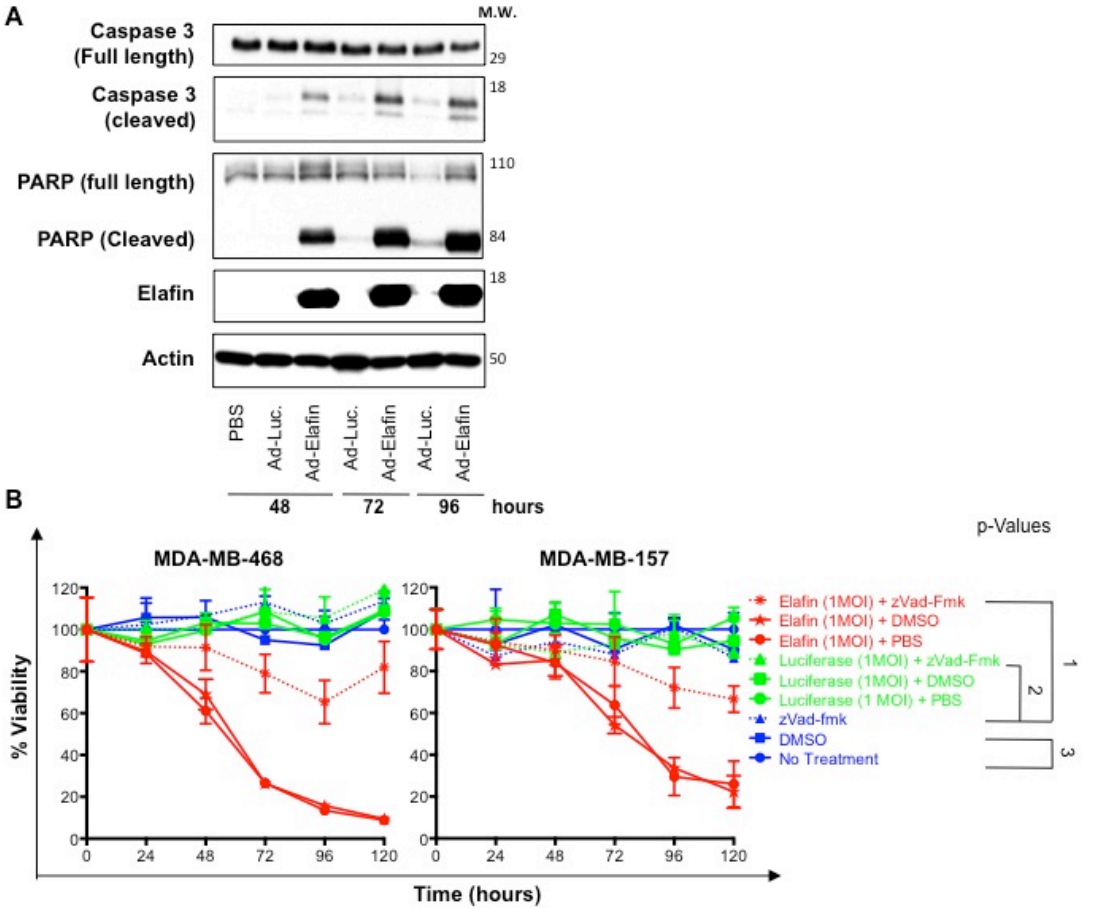


Figure 32: Expression of Elafin in Rb Negative Breast Cancer Cells Results in Apoptosis.

(A,B) MCF-7, ZR75.1, T47D, MDA-MB-157, MDA-MB-436, and MDA-MB-468 cells were treated with PBS, adenoviral luciferase, or adenoviral elafin. (A) Proliferation was assessed 48 hours post-treatment by measuring BrdU incorporation. (B) Apoptosis was assessed 72 hours post-treatment by TUNEL assay.

Inhibition of Caspase Activity Leads to Attenuation of Elafin-Mediated Apoptosis.

Caspase activation is essential to apoptotic cell death. Caspase 3 is a component of both the intrinsic and extrinsic (death receptor) apoptotic pathways. Cleavage of caspase 3 and caspase 3 target PARP are specific indicators of apoptotic cell death (Figure 33). To determine if caspase activity is increased by adenoviral elafin expression in the Rb negative cells we examined caspase 3 and PARP cleavage by western blot analysis. Treatment of the Rb deficient cell line MDA-MB-468 with adenoviral elafin dramatically increased cleavage of PARP and caspase 3, suggesting activation of caspase-dependent apoptosis (Figure 33A). To determine if adenoviral elafin-induced cell death is caspase-dependent, MDA-MB-468 and MDA-MB-157 cells were treated with adenoviral elafin in the presence of the pan-caspase inhibitor zVad-fmk. Treatment with zVad-fmk significantly reduced elafin-mediated cell death, indicating a requirement for caspase activation in elafin-induced cell death (Figure 33B). These results suggest that elafin induces caspase-dependent, apoptosis in Rb deficient breast cancer cells and not another form of cell death.



p-Values	MDA-MB-468			MDA-MB-157		
	72 hr	96 hr	120 hr	72 hr	96 hr	120 hr
1	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
2	0.0004	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
3	0.007	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Figure 33: Inhibition of Caspase Activity Leads to Attenuation of Elafin-Mediated Apoptosis.

(A) MDA-MB-468 cells were treated with PBS, adenoviral luciferase, or adenoviral elafin for 48, 72, and 96 hours. Cells were harvested and lysates were subjected to western blot analysis for full length caspase 3, cleaved caspase 3 (Asp175), PARP, and elafin. Actin, loading control. (B) MDA-MB-468 and MDA-MB-157 cells were treated with PBS, adenoviral luciferase, or adenoviral elafin in the presence of 50 μM zVad-fmk, DMSO, or PBS. Viability was measure by MTT assay every 24 hours for 120 hours. Eight replicates from two independent experiments were combined and expressed as a percentage of the PBS control. Statistical significance was calculated by the t-test for no treatment vs. elafin-alone (1), luciferase alone vs. elafin alone (2), and elafin + DMSO vs. elafin + zVad-fmk (3): displayed as a table.

MDA-MB-231 Rb Knockdown Cells are Sensitive to Elafin-Induced Apoptosis.

We next examined if Rb directly mediates the sensitivity of breast cancer cell lines to elafin-induced cytotoxicity. MDA-MB-231 cells expressing Rb shRNA (RBKD) cells were evaluated by western blot analysis. In these cells Rb is efficiently downregulated (Figure 34A; left panel). Adenoviral expression of the elafin transgene was approximately equivalent in the MDA-MB-231 RBKD and controls cell lines and similar to the level expressed by 76NE6 cells growth factor deprived for 48 hours (control) (Figure 34A; right panel). MDA-MB-231 RBKD cells demonstrate enhanced growth kinetics compared to control and parental cells (Figure 34B). MDA-MB-231 RBKD cells also demonstrate enhanced sensitivity to the adenoviral elafin-induced cytotoxicity (Figure 34B). Treatment of the MDA-MB-231 RBKD cells with adenoviral elafin resulted in a significant reduction in cell number 72 and 96 hours post-treatment when compared to MDA-MB-231 parental or shRNA control cells (Figure 34C). To validate that the decrease in cell number was due to adenoviral elafin induced apoptosis, TUNEL staining was performed. A significant increase in TUNEL positivity was detected in MDA-MB-231 RBKD cells compared to control cell lines (Figure 34D). These results illustrate a direct role for Rb deficiency in sensitizing breast cancer cells to elafin-induced apoptosis.

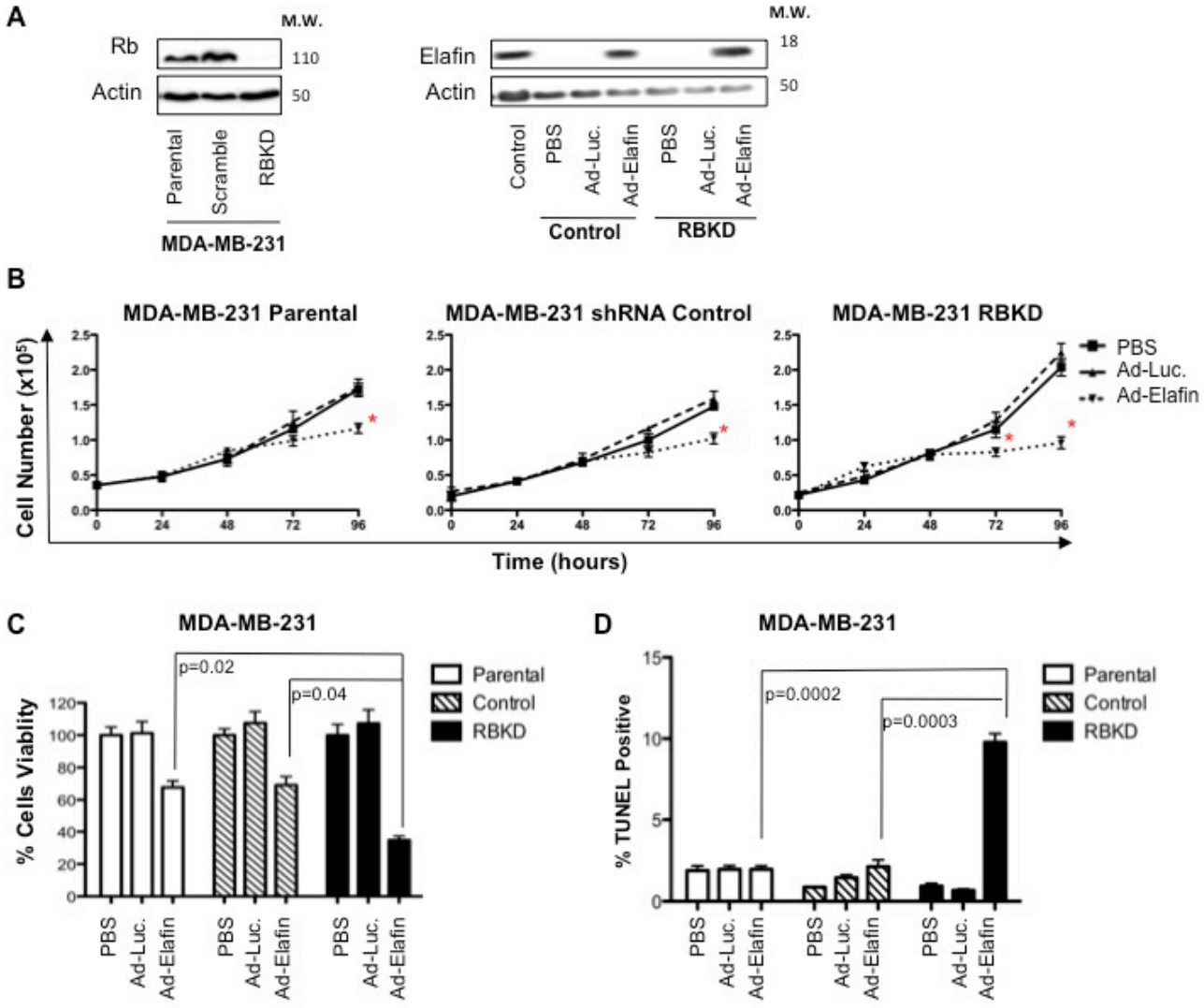


Figure 34: Knockdown of Rb in MDA-MB-231 Cells Increases Sensitivity to Elafin-Induced Apoptosis.

(A-D) MDA-MB-231 parental, shRNA control, and RBKD cell lines were treated with PBS, adenoviral luciferase, or adenoviral elafin (A) Lysates were collected at 48 hours and subjected to western blot analysis. PBS treated lysates were examined by western for Rb expression, left panel. All lysates were examined for elafin expression, right panel. Actin, loading control. (B) Growth was monitored by trypan blue exclusion test every 24 hours for 96 hours. Asterisks indicate a statistically significant difference in cell number comparing elafin treated to luciferase and PBS treated cells. (C) Cell number at 96 hours was compared between MDA-MB-231 parental, control, and RBKD cells; cell viability was calculated as a percentage of the PBS control. (D) Apoptosis was assessed 72 hours post-treatment by TUNEL assay.

Rb is Required for Elafin Induced G0/G1 Arrest in MCF-7 Cells.

Treatment of Rb positive breast cancer cell lines with adenoviral elafin causes them to undergo proliferative arrest not apoptosis (Figure 32). To further characterize cell cycle arrest in Rb positive breast cancer cells we performed DNA content analysis. Treatment of MCF-7 (Rb positive) cells with adenoviral elafin results in the accumulation of cells in the in the G1 phase of the cell cycle (Figure 35A). Rb is a critical component of the G1 checkpoint control, therefore we examined if elafin overexpression modulates CDK4 kinase activity and Rb phosphorylation in MCF-7 breast cancer cells. For these experiments MCF-7 cells were treated with PBS, adenoviral luciferase, or adenoviral elafin for the 24, 48, and 72 hours. Lysates were subjected to western blot analysis and a CDK4 immunoprecipitation kinase assays (Figure 35). Elafin treatment resulted in a decrease in the phosphorylation of Rb using phospho-specific antibodies, directed against serine 807/811, serine 780, and total phosphorylated Rb (Figure 35B). The CDK4 kinase activity was measured using GST-Rb as a substrate. CDK4 kinase activity was profoundly decreased following overexpression of elafin (Figure 35C). These results suggest that the expression of elafin in MCF-7 cells causes a G1 arrest characterized by a decreased in Rb phosphorylation, in part due to attenuation of CDK-4 kinase activity.

Next, we set out to examine if Rb downregulation in MCF-7 cells is sufficient to convert cell fate from growth arrest to apoptosis following elafin expression (as was the case in the RBKD MDA-MB-231 cells [Figure 34]). To accomplish this we treated MCF-7 cells stably expressing an shRNA vector targeting Rb with PBS, adenoviral luciferase, or adenoviral elafin and examined cell viability (Figure 36). Efficient downregulation of Rb was observed by western blot analysis in MCF-7 RBKD cells compared to control and parental MCF-7 cells (Figure 36A; left panel). Consistent expression of the elafin transgene was confirmed by western blot analysis for MCF-7 RBKD and control cells (Figure 36A; right panel). Elafin expression achieved in MCF-7 RBKD and controls was equivalent to levels observed in 76NE6 cells growth factor deprived for 48 hours (Figure 36A; right panel). MCF-7 shRNA control cells treated with adenoviral elafin demonstrated a moderate reduction in cell number at 96 hours when compared to luciferase control (Figure 36B), similar to the pattern seen in the MCF-7 parental cell

line (Figure 31A). MCF-7 RBKD cells showed no significant change in growth kinetics following treatment with adenoviral elafin or adenoviral luciferase (Figure 36C). MCF-7 RBKD cells demonstrate enhanced growth kinetics compared to controls (Figure 36C) Direct comparison of the parental, shRNA control, and RBKD cell lines (normalized to PBS control) illustrates failure of RBKD cells to arrest following administration of adenoviral elafin, compared to control and parental cells. MCF-7 RBKD cells demonstrate significantly less sensitivity to elafin-induced cell cycle arrest, as measured by BrdU incorporation (Figure 36D). This indicates that knockdown of Rb in MCF-7 cells does not sensitize cells to apoptosis, but is sufficient to attenuate cell cycle arrest.

Collectively, these results show that elafin induced growth arrest in breast cancer cells is Rb-dependent.

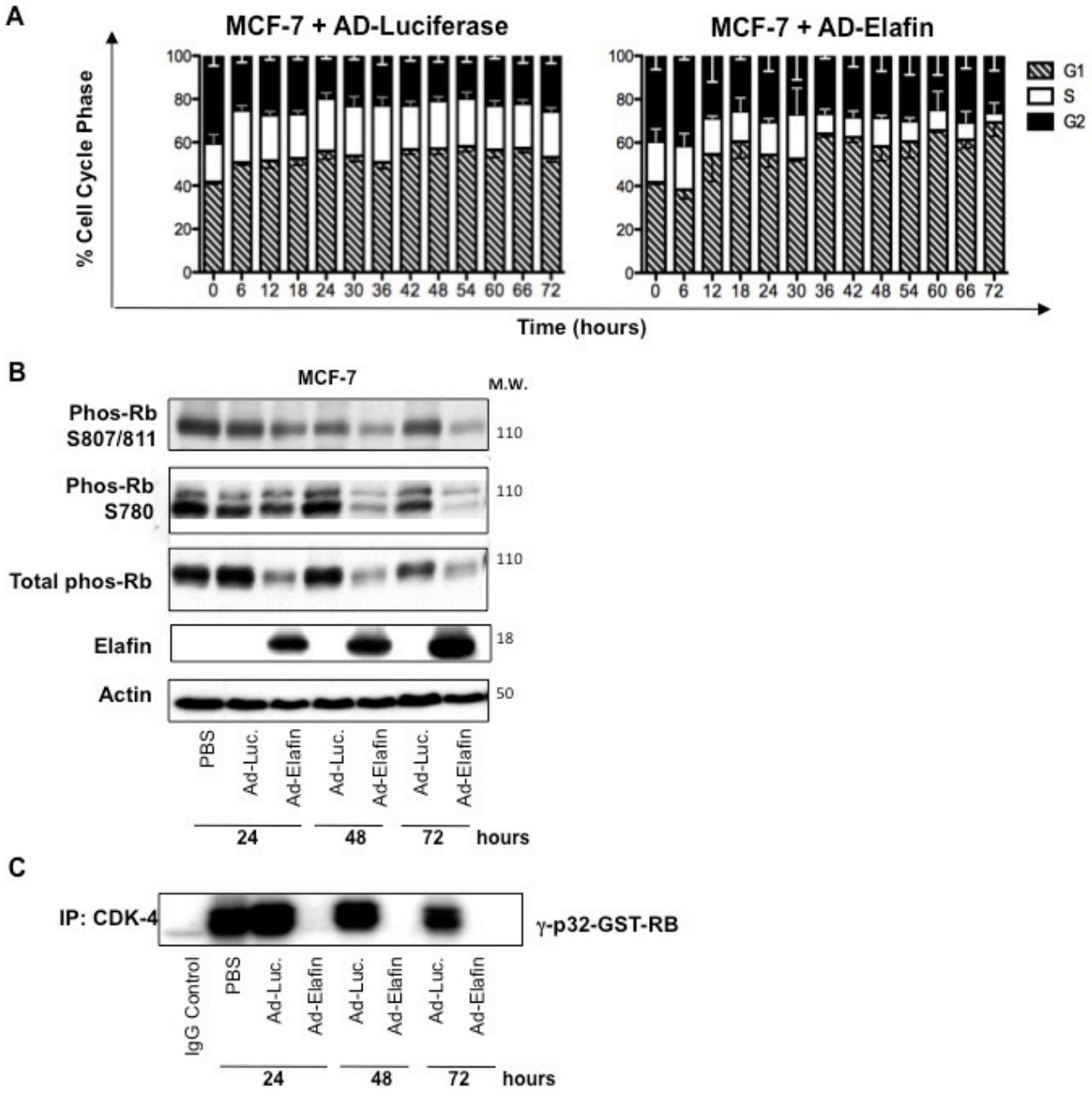


Figure 35: Expression of Elafin in MCF-7 Cells Causes a G0/G1 arrest.

(A) MCF-7 cells were infected with adenoviral luciferase or adenoviral elafin. Cells were collected every six-hours for 72-hours and subjected to DNA content analysis. (B-C) MCF-7 cells were treated with PBS, adenoviral luciferase, or adenoviral elafin. (B) Lysates were subjected to western blot analysis and probed for pRb S807/811, pRb S780, total pRb, and elafin. (C) CDK-4 was immunoprecipitated from 250 μg of protein lysates, the immunocomplex was then subjected to in vitro kinase assay using GST-Rb as a substrate.

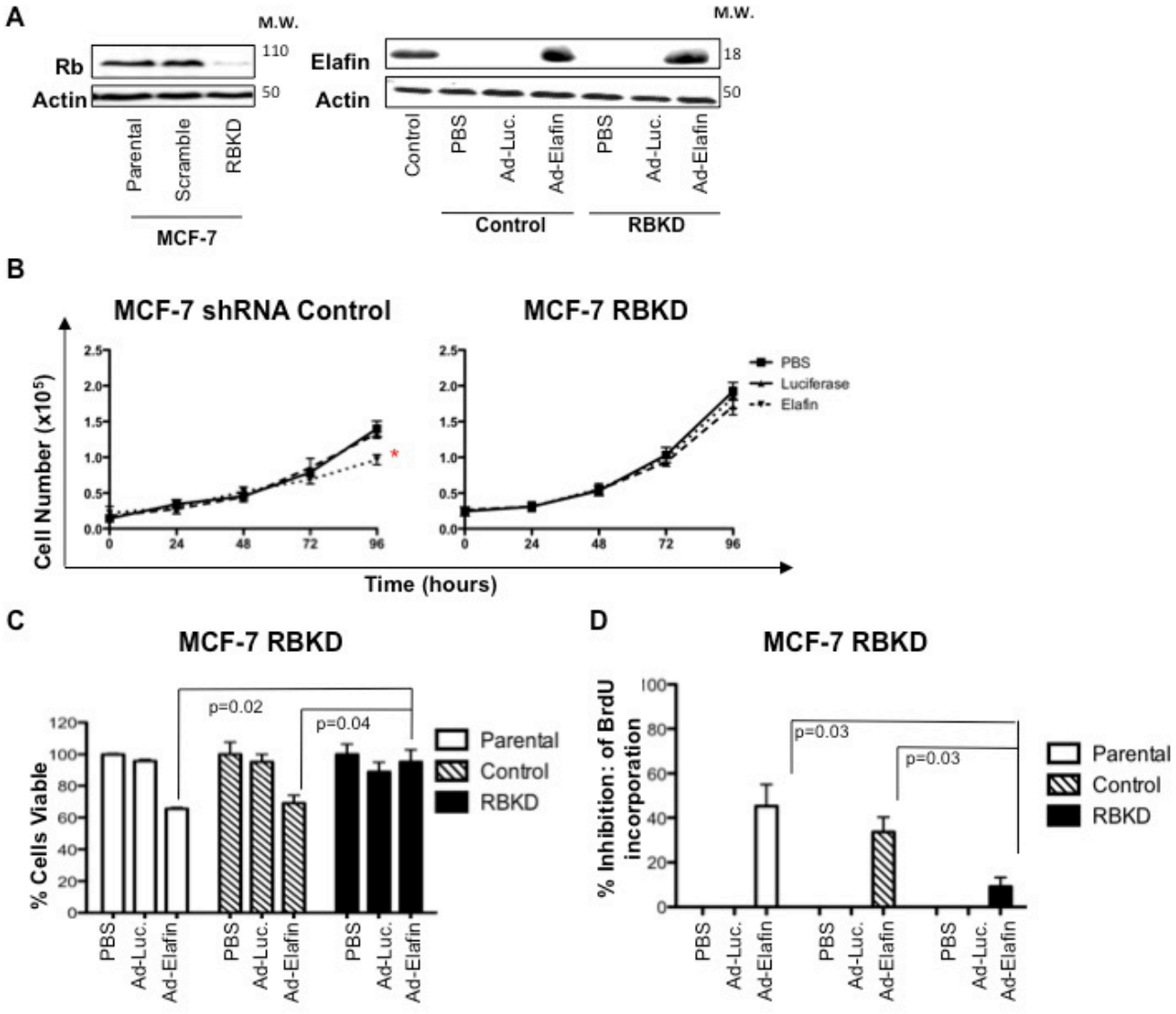


Figure 36: Rb is Required for Eafin-Induced G0/G1 Arrest in MCF-7 Cells.

(A-D) MCF-7 parental, shRNA control, and RBKD cell lines were treated with PBS, adenoviral luciferase, or adenoviral elafin (A) Lysates were collected at 48 hours and subjected to western blot analysis. PBS treated samples were examined for Rb expression, left panel. All lysates were examined for elafin expression, right panel. Actin, loading control. (B) Growth was monitored by the trypan blue exclusion test every 24 hours for 96 hours. Asterisk denotes statistical significance. (C) Cell number was compared 96 hours post-treatment between MCF-7 parental, shRNA control, and RBKD cell lines as a percentage of the PBS controls. (D) Proliferation was assessed 48 hours post-treatment by measuring BrdU incorporation. Values are expressed as percent inhibition of BrdU incorporation; values normalized to PBS and then subtracted from 100.

Overexpression of Elafin Causes Apoptosis in MCF-7 Cells Only After Knockdown of Rb and Restoration of Caspase 3.

Downregulation of Rb was shown to sensitize MDA-MB-231 cells to elafin induced apoptosis; however downregulation of Rb in MCF-7 cells failed to replicate these results. MCF-7 cells lack endogenously expressed caspase 3. Therefore we hypothesized that loss of Rb and presence of caspase 3 are both required for elafin-induced apoptosis. To address this hypothesis, we first confirmed that elafin expression fails to induce apoptosis following reconstitution of caspase 3 alone. Western blot confirmed the expression of caspase 3 in stably transfected MCF-7 cells (Figure 37A, left panel). MCF-7 caspase 3 reconstituted cells were treated with PBS, adenoviral luciferase, or adenoviral elafin. Western blot analysis confirms equivalent expression of elafin (Figure 37A). No significant increase in apoptotic cell death, measured by TUNEL assay, was seen in MCF-7 caspase 3 or MCF-7 RBKD cells in response to adenoviral elafin (Figure 37B).

To directly examine if Rb downregulation and presence of caspase 3 are co-requirements for apoptosis in MCF-7 cells following elafin expression, we established clones of MCF-7 RBKD and MCF-7 shRNA control cell lines expressing either pcDNA3.1-empty vector or pcDNA3.1-caspase 3. Knockdown of Rb, stable expression of caspase 3, and expression of the elafin transgene were verified by western blot analysis (Figure 37C,D). Treatment of MCF-7 RBKD cells expressing caspase 3 with adenoviral elafin caused a severe reduction in cell viability (Figure 38A). The same experiment was performed in MCF-7 shRNA control cells line, however in these cells reconstitution of caspase 3 did not lead to a significant decrease in viability compared to controls. Comparison of cell viability at 120 hours post treatment demonstrates that elafin mediated loss of cell viability in MCF-7 RBKD clones expressing caspase 3 is significantly different from all other MCF-7 cell lines examined (Figure 38B). TUNEL assay reveals a significant increase in the percentage of cells undergoing apoptosis in MCF-7 RBKD expressing caspase 3 versus controls (PCDNA + Ad-luc, PCDNA+Ad-Elafin or CASP 3 + Ad-Luc) (Figure 38C).

These results indicate that loss of Rb and the reconstitution of caspase 3 are both required for elafin-induced apoptosis in the MCF-7 cell line.

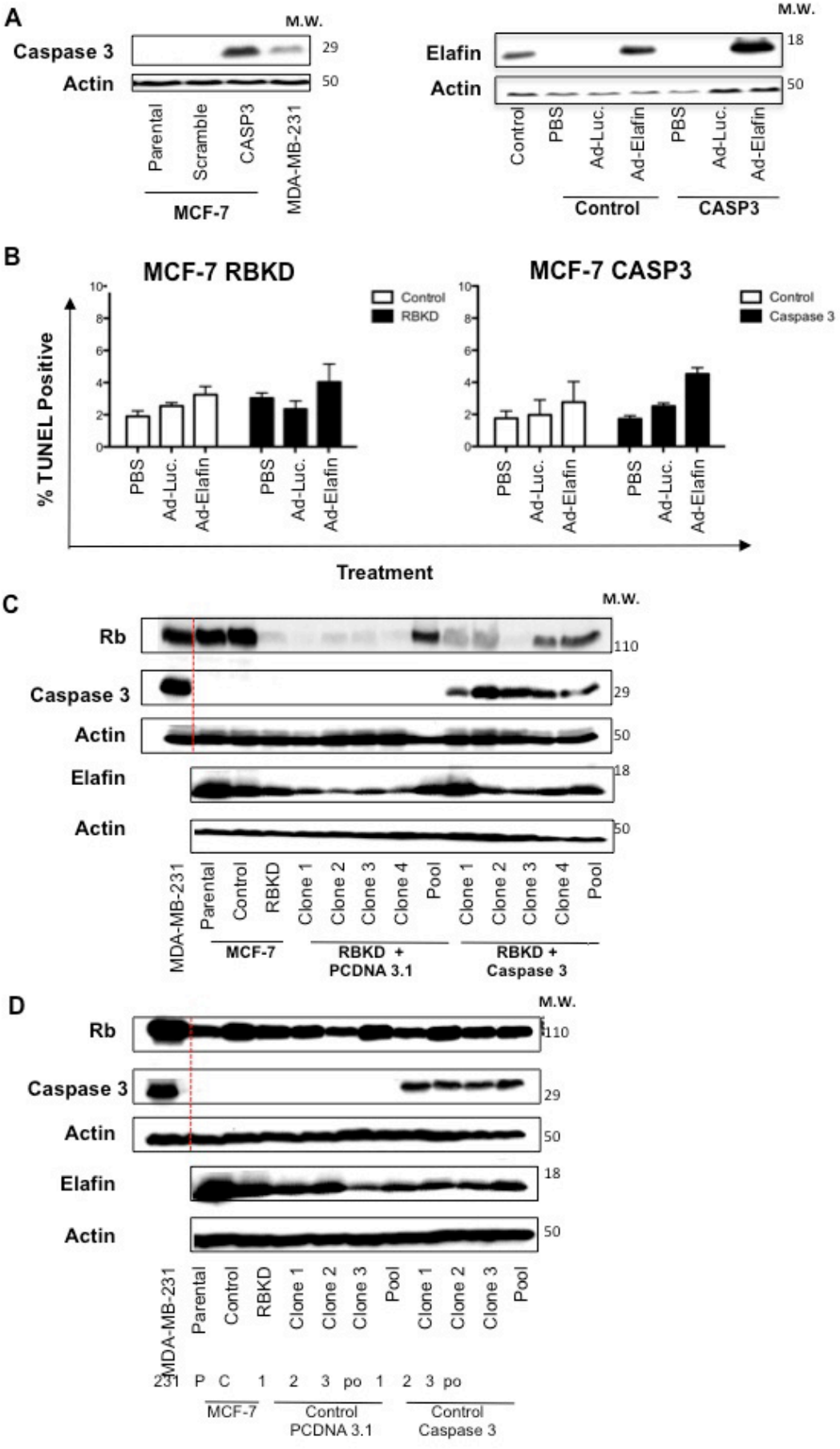

A
M.W.
Caspase 3
Actin
29
50
Parental
Scramble
CASP3
MDA-MB-231
MCF-7
M.W.
Elafin
Actin
18
50
Control
PBS
Ad-Luc.
Ad-Elafin
PBS
Ad-Luc.
Ad-Elafin
Control
CASP3
B
MCF-7 RBKD
Control
RBKD
MCF-7 CASP3
Control
Caspase 3
% TUNEL Positive
PBS
Ad-Luc.
Ad-Elafin
Treatment
C
M.W.
Rb
Caspase 3
Actin
Elafin
Actin
110
29
50
18
50
MDA-MB-231
Parental
Control
RBKD
Clone 1
Clone 2
Clone 3
Clone 4
Pool
MCF-7
RBKD + PCDNA 3.1
RBKD + Caspase 3
D
M.W.
Rb
Caspase 3
Actin
Elafin
Actin
110
29
50
18
50
MDA-MB-231
Parental
Control
RBKD
Clone 1
Clone 2
Clone 3
Pool
231
P
C
1
2
3
po
MCF-7
Control PCDNA 3.1
Control Caspase 3

Figure 37: Adenoviral Elafin does not Induce Apoptosis in MCF-7 Rb Knockdown Cells.

(A) Lysates from MCF-7 pcDNA3.1 empty vector and pcDNA3.1-caspase 3 stable clones were subjected to western blot analysis for caspase 3. Actin, loading control (left panel). Lysates from MCF-7 pcDNA3.1 empty vector and pcDNA3.1-caspase 3 were subjected to western blot analysis for elafin 48 hours after treatment with PBS, adenovirus luciferase, or adenovirus elafin. Actin, loading control (right panels). (B) MCF-7 pcDNA3.1 empty vector, pcDNA3.1-caspase 3, RbKD, and shRNA control cells were treated with PBS, adenovirus luciferase, or adenovirus elafin. Apoptosis was assessed by TUNEL assay. (B) MCF-7 RBKD cells were transected with either pcDNA3.1 empty vector or pcDNA3.1-caspase 3 and selected in puromycin. Cells were treated with adenoviral elafin. Lysates were subjected to western blot analysis. Untreated cells were used to assess the levels of Rb and caspase 3, reproducibility of transgene expression was assessed by western blot for elafin.

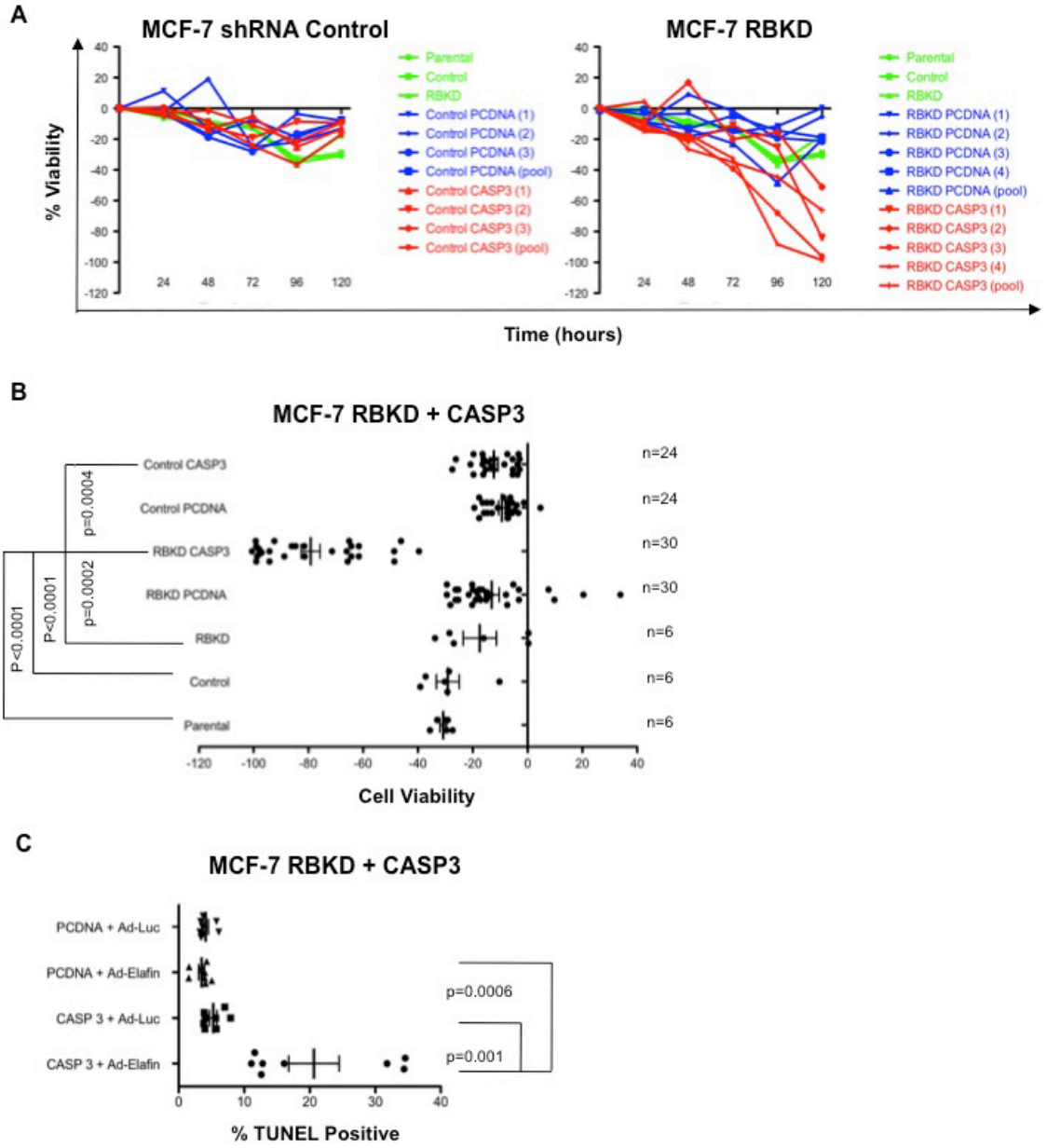

A
MCF-7 shRNA Control
MCF-7 RBKD
% Viability
Time (hours)
Parental
Control
RBKD
Control PCDNA (1)
Control PCDNA (2)
Control PCDNA (3)
Control PCDNA (pool)
Control CASP3 (1)
Control CASP3 (2)
Control CASP3 (3)
Control CASP3 (pool)
RBKD PCDNA (1)
RBKD PCDNA (2)
RBKD PCDNA (3)
RBKD PCDNA (4)
RBKD PCDNA (pool)
RBKD CASP3 (1)
RBKD CASP3 (2)
RBKD CASP3 (3)
RBKD CASP3 (4)
RBKD CASP3 (pool)
B
MCF-7 RBKD + CASP3
Control CASP3
Control PCDNA
RBKD CASP3
RBKD PCDNA
RBKD
Control
Parental
p=0.0004
p=0.0002
P<0.0001
P<0.0001
n=24
n=24
n=30
n=30
n=6
n=6
n=6
Cell Viability
C
MCF-7 RBKD + CASP3
PCDNA + Ad-Luc
PCDNA + Ad-Elafin
CASP 3 + Ad-Luc
CASP 3 + Ad-Elafin
p=0.0006
p=0.001
% TUNEL Positive

Figure 38: Overexpression of Elafin Causes Apoptosis in MCF-7 Cells Only After Knockdown of Rb and Restoration of Caspase 3.

(A) Cell viability was measured by MTT assay every 24 hours for 120 hours and calculated at each time point by normalizing values from luciferase and elafin treated cells to PBS control then plotting the difference between the viability of elafin and luciferase (i.e. elafin effect – viral effect). (B,C) MCF-7 control shRNA background cells expressing either pcDNA3.1 empty vector or pcDNA3.1-caspase 3. These cells were assayed in the same manner as panels B and C. (C) The viabilities measured at 120 hours in the cell lines generated from the MCF-7 RBKD and MCF-7 control shRNA as well as the parental cell lines were pooled and statistically compared. (C) Apoptosis was measured by TUNEL assay.

DISCUSSION

Elafin is expressed in HMECs, but it is transcriptionally downregulated in tumor derived cell lines (24). In chapter 2, the data presented demonstrated that elafin was highly upregulated in growth factor deprived, G0-HMECs (Figure 15) and is required for maintenance of G0-arrest (Figure 21). In the absence of Rb, growth factor deprived HMECs failed to arrest in G0 and did not express elafin at levels comparable to Rb-expressing HMECs (Figure 18). In this chapter, adenoviral-mediated expression of elafin was used to determine if elafin expression alone is sufficient to induce growth arrest in normally proliferating Rb-expressing HMECs (76NE6 and 76NF2V) and Rb-deficient (76NE7) HMECs. In this experiment, adenoviral-elafin failed to induce growth arrest in Rb-expressing HMECs. However, in HMECs lacking Rb, adenoviral-elafin surprisingly caused apoptotic cell death (Figure 30). This Rb-dependent apoptotic effect led to the hypothesis that tumor cells with a deregulated Rb pathway could also be forced to undergo apoptosis following elafin expression. Indeed, we observed that the expression of elafin could induce apoptosis in Rb-negative cells (Figure 32). We also found that Rb-positive breast cancer cells are growth inhibited in response to elafin expression (Figure 32). In these cells, elafin expression led to the downregulation of CDK4 activity and a reduction in Rb phosphorylation (Figure 35). Critically, Rb-positive MDA-MB-231 cells, which are insensitive to adenoviral elafin-induced apoptosis, could be converted to a sensitive phenotype by downregulation of Rb alone (Figure 34).

Of note, the Rb-negative cell lines used in this work were all of the basal-like subtype and the Rb-positive cell lines were all of the luminal subtype. Rb-loss is rare in the luminal subtypes of breast cancer (Figures 4 and 5), however it occurs with greater frequency in the basal-like subtype of breast cancer (Figure 7). To exclude the possibility that adenoviral elafin-induced cytotoxicity is exclusive to the basal-like subtype, MCF-7 (luminal subtype) cells were converted to an elafin-sensitive phenotype (Figure 38). MCF-7 cells were not sensitized to the apoptotic effect of adenoviral elafin following Rb knockdown alone (Figure 37), as was the case with MDA-MB-231 cells (Figure 34). Caspase activity is required for adenoviral elafin induced apoptosis (Figure 33). MCF-7 cells lack caspase 3 (Figure 37). Restoration of caspase 3 in MCF-7 Rb knockdown cells was sufficient to sensitize these cells to adenoviral elafin induced

apoptotic cell death, suggesting that the activity of elafin in breast cancer cell lines is not dictated by breast cancer subtype. Rb-status and an intact caspase-dependent apoptotic cascade are major determinate of sensitivity to elafin-induced apoptosis versus growth arrest. Taken together, the results presented here clearly indicate that elafin has tumor suppressive properties in breast cancer cell lines similar to results reported for the serine protease inhibitors SLPI and maspin (747, 751-753, 761).

Following publication of the work presented here (678), adenoviral elafin was shown to have therapeutic efficacy following intratumoral injection of MDA-MB-468 xenograft tumors (25). Adenoviral elafin treated MDA-MB-468 xenograft tumors demonstrate a significant growth delay compared to adenoviral luciferase or PBS treated tumors. The survival of adenoviral elafin treated, tumor-bearing mice was significantly extended. Approximately 20% of the tumors were completely eradicated by adenoviral elafin treatment (25).

Elafin downregulation has also been observed in melanoma. Elafin re-expression using a tet-inducible system resulted in apoptotic cell death in melanoma cells, but not normal melanocytes. In this system p53 was required for the elafin-induced cell death (27). In the work presented here, apoptotic cell death is p53 independent since all of the breast tumor cell lines (MDA-MB-468, MDA-MB-157, MDA-MB-436) sensitive to elafin-induced apoptosis express mutant p53. Induction of elafin expression in melanoma xenograft tumors significantly reduced tumor size (27).

The majority of breast tumors demonstrate elafin downregulation (Figure 10). Approximately one-quarter of all breast tumors exhibit homologous loss or mutation of Rb (768, 769). Rb loss is associated with aggressive breast cancer subtypes, especially basal-like tumors (Figure 7) (770, 771). The basal-like subtype of breast cancer are resistant to currently available targeted therapies (i.e. anti-estrogens and anti-HER2) (427, 772). A therapy capable of specifically targeting breast tumors with Rb loss may have significant clinical utility in highly aggressive breast tumors. Elafin represents a candidate therapeutic capable of specifically targeting tumor cells with disruption of the G1 checkpoint with no toxicity in normally dividing cells.

Chapter 4: Neutrophil Elastase in Basal-like Breast Cancer Progression

INTRODUCTION

TAN and Tumorigenesis

Inflammatory signaling and the presence of leukocytes within the tumor microenvironment are critical components of tumor progression (9, 10). Early tumor microenvironment studies largely ignored the neutrophil component of the inflammatory infiltrate due to doubts that such a short-lived cell type could dramatically impact tumorigenesis (423).Tumor-derived chemokines drive the constant replenishment of neutrophils in the tumor microenvironment. Chemokines are produced directly by tumor cells as a consequence of oncogene-induced (e.g. K-Ras and Myc) NF-κB pathway activation (368, 407) and by activated fibroblasts subjected to hypoxia, chemotherapeutics, and other cellular stresses (373, 375).

Relatively few studies have examined tumor-associated neutrophils (TAN) in human cancer. In renal cell carcinoma, TAN (CD66b-positive) were prognostic of short recurrence-free survival (RFS). In the presence of TAN, patients demonstrated a five-year RFS of 53%, however in the absence of TAN patients had a five-year RFS of 87%. In multivariate analysis, TAN were an independent prognostic marker of disease-specific survival (DSS) and overall survival (OS) (410). In human gliomas, the concentration of TAN (CD15-positive and myeloperoxidase-positive) correlate with high tumor grade (411). High levels of neutrophils (scored based on H&E) in the bronchoalveolar lavage fluid from patients with bronchioloalveolar carcinoma were associated with high levels of IL-8, NE, and poor DSS (407). Although not commonly observed in pancreatic tumors, TAN (scored based on H&E staining) were associated with the aggressive micropapillary and undifferentiated subtypes (412). High neutrophil levels in the peripheral blood were associated with poor outcome in metastatic melanoma and renal cell carcinoma patients (408, 409).

TAN contribute growth factors (i.e. HGF), cytokines (i.e. Oncostatin M, TNFα, IL1-β, IL-6, and IL-12), reactive oxygen species (ROS), and proteases (i.e. NE, PR3,

CG, MMP-8, and MMP-9) to the tumor microenvironment (345, 418-421). In mouse models, neutrophil depletion using Gr-1 (LY-6G) antibodies or blockade of neutrophil recruitment using antibodies against Cxcr2, profoundly inhibited tumor growth, angiogenesis, and metastasis in mouse tumor models (415-417). These studies suggest a pro-tumorigenic role for TAN in human cancer. TGF-β blockade forced TAN to assume an anti-tumor phenotype, however little evidence exists of a role for TAN in anti-tumor immunity in the absence of therapeutic manipulation (422, 423).

NE and Tumorigenesis

Several groups demonstrated that high levels of NE (measured by ELISA) were independently prognostic of OS, metastasis-free survival, DSS, and RFS in multivariate analysis of breast cancer patients (12, 599-601). High NE expression demonstrated a negative correlation with tumor ER and PR status. In ER-positive tumors the mean level of NE was 5.8 ng/mg of tissue compared to 14.9 ng/mg in ER-negative tumors. In PR-positive tumors the mean level of NE was 6.0 ng/mg compared to 9.1 ng/mg in PR-negative tumors (12). Triple-receptor negative breast cancer (TNBC) highly expresses chemokines critical to the recruitment and activation of TAN (773-776), indicating that the recruitment of neutrophils is highest in TNBC and that these are the tumors with the greatest concentration of NE. However, NE may also play a role in the outcome of patients with ER-positive tumors. High levels of NE are associated with a poor response rate to tamoxifen, shorter progression-free survival, and poor post-relapse free survival. In multivariate analysis, NE is an independent predictive marker of response to tamoxifen (599).

Sivelestat, a pharmacological inhibitor of NE, was able to reduce the proliferation, motility, and chemotaxis of the pancreatic cancer cell line Capan-1 *in vitro* (603). In a mouse xenograft model of non-small cell lung cancer using the EBC-1 and PC-1 cell lines, sivelestat attenuates tumor proliferation (606). Sivelestat also inhibited spontaneous metastasis of EBC-1 xenograft tumors (607). Beige mice are deficient in NE (608). Cohorts of NE-deficient and control mice were created for the study of NE in tumorigenesis by crossing beige mice with SKH 1 hairless mice. Following ultraviolet irradiation, NE-expressing control mice developed an average of 10 tumors per mouse after 20-weeks, while NE-deficient mice developed an average of only 0.4 tumors per

mouse over the same period. In the same system, benzopyrene exposure resulted in the formation an average of 7 tumors per control mouse and an average of only 0.2 tumors per NE-deficient mouse. Pharmacological inhibitors of NE, 2,4,6-trinitrochlorobenzene and oxazolone were able to attenuate the development of skin tumors following ultraviolet irradiation of SKH 1 mice (609). NE knockout mice were developed to understand the normal role of NE in immunity and inflammation (500). NE knockout in the transgenic *loxP*-Stop-*loxP* K-ras^{G12D} mouse model of lung cancer dramatically reduced tumor burden compared to control. NE knockout significantly extended survival in *loxP*-Stop-*loxP* K-ras^{G12D} mice. Over a 30 weeks period following administration of adenoviral Cre all of the NE+/+ genotype mice had reached the limit of tumor burden, however none of the NE-/- mice has reached the same limit. NE knockout dramatically reduced tumor growth. NE-mediated degradation of IRS-1 resulted in the activation of the PI3K-Akt pathway and may be responsible for enhanced tumor growth (542).

Hypothesis and Central Findings

The hypothesis tested in this chapter states that NE is essential to the growth and progression of breast cancer. Immunohistochemical analysis of NE in breast tumors revealed that high levels of NE-expressing, TAN are associated with TNBC and are prognostic of poor recurrence-free survival (RFS). ERK-catalyzed phosphorylation of p90RSK (T359/S363) and Rb phosphorylation (S807/811) were significantly enriched in NE-positive tumors. We generated C3(1)TAg x NE-/- mice to directly examine the role of NE in TNBC development. Compared to C3(1)TAg x NE+/+ littermates, the C3(1)TAg NE-/- mice showed decreased tumor growth and proliferation. Although NE is implicated in inflammation, no statistically significant difference in the expression of inflammatory cytokines, neutrophil, or macrophage infiltration was observed.

MATERIALS AND METHODS

The methodology for preparation of protein lysates, western blot analysis, growth curves, and immunohistochemistry are detailed in chapter two, modifications, additional reagents, and additional protocols are described here.

Antibodies

Primary antibodies used for western blot (WB) and immunohistochemistry (IHC):

Antibody	Species	Clone	Company	Application
Neutrophil Elastase	Mouse monoclonal	NP57	DAKO	IHC
Egr-1	Rabbit polyclonal		Santa Cruz Biotechnology	WB
Phospho-p90RSK site Thr359/Ser363	Rabbit polyclonal		Cell Signaling Technology	WB
BrdU	Rat monoclonal	BU1/75 (ICR1)	AbD Serotec	IHC
Ly6G (Gr-1)	Rat monoclonal	clone 1A8	BD Biosciences	IHC
F4/80	Rat monoclonal	6A545	Santa Cruz Biotechnology	IHC
Irs-1	Mouse monoclonal	4.2.2	Millipore	WB
Phospho-Akt	Rabbit monoclonal	D9E	Cell Signaling Technology	WB
IκB	Rabbit monoclonal	44D4	Cell Signaling Technology	WB
Phospho-GSK3β site Ser9	Rabbit polyclonal		Cell Signaling Technology	WB
SV40 large T-antigen	Rabbit polyclonal		Santa Cruz Biotechnology	WB

Immunohistochemistry

IHC analysis was performed as described in chapter two. The antigen retrieval was omitted for NE-IHC because it destroys the epitope recognized by the antibody. For IHC detection of BrdU, Ly6G, and F4/80 antibodies the biotin conjugated rabbit anti-rat

secondary antibody (Abcam) was substituted for the secondary goat anti-mouse antibody included on the Vectastain kit (Vector).

Patient Samples

For analysis of NE in breast tumors, 306 patients with stage I-III breast cancer were enrolled in a prospective study between January 2000 and June 2010 (MD Anderson lab protocol 00222). Fresh frozen tumor tissue and formalin-fixed, paraffin-embedded tissue blocks were obtained from eligible participants. The median age of the patient population was 56 years (range: 26-92 years) and median follow up was 6.2 years. Complete clinical and follow-up data was obtained by review of the patients' files. The MD Anderson Institutional Review Board approved the use of all patient derived tissues.

Reverse Phase Protein Array (RPPA)

Tumor tissue was thawed and homogenized using a micromincer (BioSpec) and prepared using the same protocol as western blot analysis described in chapter two. RPPA analysis performed by the Functional Proteomics core facility at UT MDACC has been previously described (777). Patient tumors were homogenized, lysed via sonication, and cleared by ultracentrifugation; as previously described for western blot analysis (678). Protein concentration was determined by Bradford assay and the lysates were aliquoted in SDS containing sample buffer at a concentration of 1µg/µL. Protein samples were serially diluted and arrayed on nitrocellulose coated slides (Grace Biolab) using an Aushon 2470 Arrayer (Aushon BioSystems). The slides are probed with different primary antibodies, followed by the appropriate biotin-conjugated secondary antibody. The signal was amplified using the Catalyzed Signal Amplification System (DAKO) and detected using DAB (DAKO). The slides were scanned with ImageQuant (Molecular Dynamics) and spot intensity was determined by MicroVigene software (VigeneTech Inc.). Protein abundance was defined by supercurve fitting, a logistic model developed by the Dept. of Bioinformatics and Computaional Biology UT MDACC for the relative quantification of each sample, and normalized for protein loading.

RPPA Antibodies

14-3-3 ζ
14-3-3 β
4EBP1
4EBP1 pS65
4EBP1 pT37
A-Raf pS299
αTubulin
αβ-Crystallin
ACC pS79
ACC1
AIB1
AKT
AKT pS473
AKT pT308
AMPK pT172
AMPK
Annexin
ARV
ATM
ATM pS198
ATR pS423
ATRIP
Beta-Catenin
Beta-catenin pS33
B-RAF
BAD pS112
BAK
BAX
BClX
BClXL
BCl2
BCl2
BCl2 pS70
BID
BIM
BRCA1
BRCA2
cJun pS73
cKIT
cMyc
cMyc pT58
cRAF
Caspase 7 cleaved
Caspase 3 cleaved
Caveolin
CD20
CD31
CD86
CHK1
CHK1 pS345
CHK2
Collagenase VI
Cyclin B1
Cyclin D1
Cyclin E1
Cyclin E2
ECadherin
EGFR
EGFR pY1173
EGFR pY992
eIF4E
ELK1 pS383
ER pS167
ERα pS118
ERK2
ETV6
FAKC
FGFR1
FGFR2
Fibronectin
Fortilin
FOXM1
FOXO3a
FOXO3a pS318
GATA3
GSK pS9
GSK3 αβ
GSK3 αβ pS21
GYS1
GYS1 pS640
HER2
HLA-E
HSP27
HSP70
IGFBP2
IGFRb
IRS1
IRS1 p307
JNK pT183
KU80
MAPK pT202V
MEK1
MEK1/2 pS217
MGMTV
MMECD10
MRE11
MSH2
mTOR
N-Cadherin
NFκB p65 pS536
p21
p27
p38
p38_pT180
p53
p70S6K
p70S6K pS371
p70S6K pT389
p90RSK
p90RSK T359
PAI1
PARP cleaved
PAX2
PCNA
PDK1
PDK1 pS241
PI3K p110α
PKCE pT40
PKCα
PKCα pS657
PKCδ pS644
PLCR2 pY759
PLK1
PR
PTCH
PTEN
Rab25
RAD51
Rb
Rb pS807/811
S6
S6 pS235
S6 pS240
SHC pY317
SMAD1
SMAD3
SMAD3 pS423
SMAD4
SMAD6
SRC
SRC pY527
STAT3
STAT3 pS727
STAT3 pY705
STAT5
STAT5 pY694
STAT6 pY641
STAT6 pY641
Stathmin
SYK
TAU
TAZ
TAZ pS89
Telomerase
TSC2
TSC2 pT1462
VASP
VEGFR2
XRCC1
YAP
YAP pY127
YBI
YBI pS112
YKL40

Mice

The generation of C3(1)Tag (778) and NE knockout (500) mice were previously described. Both were maintained in the FVB/N background. Experimental cohorts of C3(1)Tag x NE-/- and C3(1)Tag x NE+/+ virgin female mice were monitored for tumor development by biweekly palpation. Tumor growth rate was analyzed by bi-weekly measurement of two orthogonal tumor diameters with a Vernier caliper. Mice were sacrificed when tumor reached the 1.5 cm along greatest axis according to institutional guidelines. Several mice were sacrificed at two months of age to assess tumor progression. Two hours prior to sacrifice the mice were injected intraperitoneally with 100 mg BrdU/kg body weight. The contralateral mammary gland was fixed in formalin and paraffin embedding for IHC analysis. The tumor was excised, half was fixed in formalin and paraffin embedding for IHC analysis and the other half was snap frozen in liquid nitrogen for protein and mRNA extraction.

RESULTS

NE-Positive TAN Predict Recurrence in Breast Cancer Patients and Correlate with the Phosphorylation of Erk-Effector p90RSK and Rb.

To understand the role of NE in patients with invasive breast cancer, the correlation between NE expression, patient outcome, clinicopathological parameters, and intracellular signaling pathways was examined in breast tumor samples. For this study, we performed immunohistochemical staining for NE and elafin on 305 breast tumors prospectively collected from patients at The University of Texas MD Anderson Cancer Center for the examination of predictive markers. This patient cohort had advantages compared to the TMA cohort presented in Chapter 2. First, full tumor blocks were available, allowing our pathologists to examine representative areas of tumor epithelium and include the peritumoral area, where TAN often localize. Second, fresh frozen tissue was available from the majority (276/305) of patients, allowing us to perform RPPA analysis.

We first validated the finding presented in Chapter 2 that elafin is downregulated in invasive breast tumors. As in Chapter 2, elafin expression was classified as at or above the expression level in normal breast epithelium (elafin score 6-8) or downregulated compared to the expression level in normal breast epithelium (elafin score 0-5) (Figure 39A). This analysis revealed that the percentage of tumors with elafin downregulation in this patient cohort was almost identical to the percentage of tumors with elafin downregulation in the TMA cohort presented in Chapter 2 (Figure 39B).

Immunohistochemical analysis of NE (Figure 40A) revealed the presence of TAN in 118 of 305 (39%) tumors examined. Positivity was defined as greater than 5 TAN per high-power field; only NE-positive neutrophils within the tumor area were counted. Breast cancer patients with high levels of TAN, defined as greater than 15 TAN per high-power field, were present in 45 of 305 tumors (15%) and correlated with a significantly poorer RFS than tumors with low levels of TAN or no TAN (Figure 40B). High levels of TAN were significantly associated with TNBC, high tumor grade, and recurrence on univariate analysis (Table 9). Cox proportional hazards analysis revealed

that high levels of TAN were independently prognostic of RFS and were associated with a hazard ratio of 2.4 (95% CI, 1.1-5.5) for RFS (Table 10).

The correlation between the NE-positive infiltrate and cancer cell signaling pathways was examined by subjecting tumor lysates from the 276 patients who had fresh frozen tissue available for RPPA analysis (Figure 41). Comparison of breast tumors positive for TAN (107/276) and breast tumors negative for TAN (169/276) using the Mann-Whitney *U*-test revealed differences in the abundance of several signaling intermediates (Table 11). High levels of p90RSK phosphorylated at threonine 359/serine 363 (phosphorylation catalyzed by ERK) and Rb phosphorylated at serine 807/811 were strongly associated with the presence of NE-positive TAN (Figure 42A).

To determine if NE can induce phosphorylation of p90RSK and Rb, 76NE6 cells and the breast cancer cell lines MDA-MB-231 and MDA-MB-157 were cultured in the absence of growth factors for 24 hours and stimulated with 10 nM NE. We observed dose-dependent increases in cell proliferation in both tumor cell lines and 76NE6 HMECs 48 hours after addition of NE (Figure 42B). MDA-MB-231, MDA-MB-157, and 76NE6 cells were also harvested 30 and 120 minutes after NE addition. Western blot analysis revealed increased phosphorylation of p90RSK T359/S363 and Rb S807/811, especially in MDA-MB-157 and 76NE6 cells (Figure 42C). NE stimulation also enhanced phosphorylation of ERK and resulted in EGR1 upregulation in these cell lines (Figure 36C). Both ERK and Rb phosphorylation were largely saturated in MDA-MB-231 cells; however, addition of NE modestly enhanced phosphorylation of p90RSK and still resulted in upregulation of EGR1 (Figure 42C).

NE-positive TAN were prognostic of poor RFS, associated with an aggressive tumor phenotype, and correlated with the phosphorylation of p90RSK and Rb. Exogenous NE activates the ERK signaling pathway in immortalized HMECs and breast cancer cell lines.

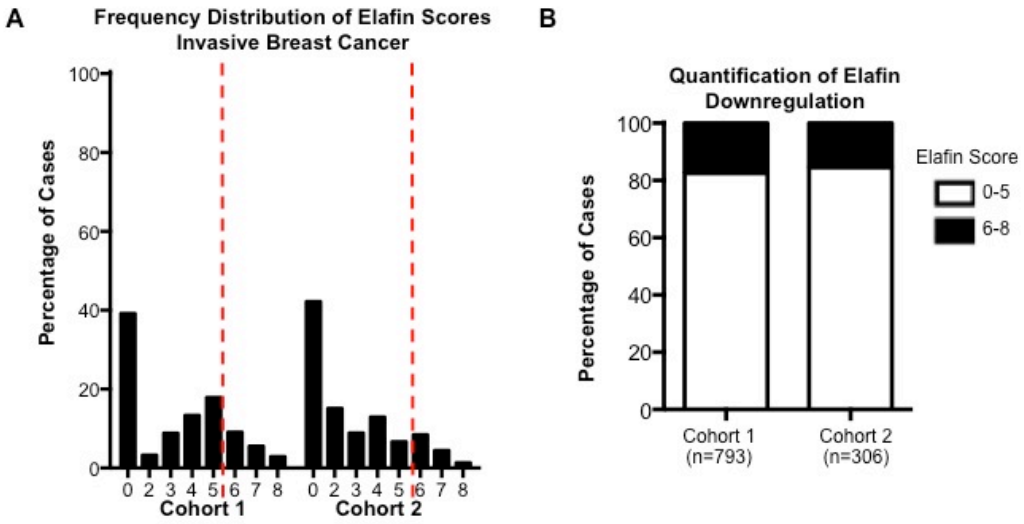


Figure 39: Comparison of Elafin Expression between Breast Cancer Patient Cohorts.

(A,B) Elafin expression was examined by IHC in the TMA breast cancer patient cohort presented in chapter 2 (Cohort 1) and in the non-TMA breast cancer patient cohort described in this chapter (Cohort 2). (A) Frequency distribution illustrating the percentage of cases falling into each categorical score over the range 0-8 for breast cancer patient cohorts. (B) Quantification of elafin downregulation in the two breast cancer patient cohorts. An elafin score of 6-8 denotes cases at or above the elafin expression level observed in the normal breast epithelium, while an elafin score of 0-5 denotes downregulation.

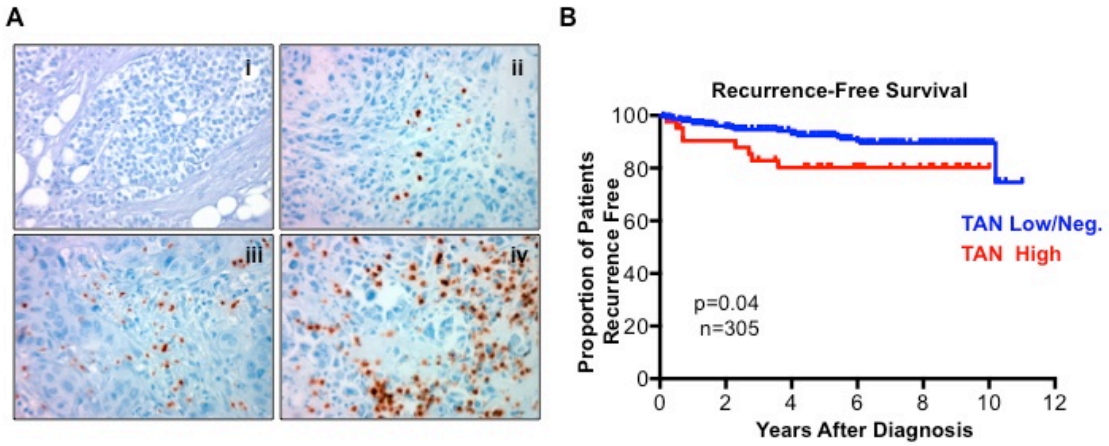


Figure 40: NE-Positive TAN Predict Recurrence in Breast Cancer Patients

(A) Representative photomicrographs of NE immunohistochemical staining of invasive breast carcinoma with negative (i), low (ii), and high (iii and iv) levels of TAN. (B) Kaplan-Meier plot of RFS in breast cancer patients segregated by expression of TAN, low/negative versus high.

Factors	Tumor Associated Neutrophils		p-value
	Negative	Positive	
	n=260	n=45	
Age at diagnosis, year			0.3
Mean	56.7	54.6	
Median (range)	56 (26-92)	55 (34-82)	
Stage			0.7
I	127 (48.9)	22 (48.9)	
IIA	108 (41.5)	17 (37.8)	
IIB	25 (9.6)	6 (13.3)	
Unknown	0	0	
ER			<0.0001
Positive	217 (84.1)	21 (47.7)	
Negative	41 (15.9)	23 (52.3)	
Unknown	0	1 (<1)	
PR			<0.0001
Positive	182 (70.5)	17 (38.6)	
Negative	76 (29.5)	27 (61.4)	
Unknown	0	1 (<1)	
Her-2			0.4
Positive	41 (16.0)	4 (9.1)	
Negative	215 (84.0)	40 (90.9)	
Unknown	2	1(<1)	
TNBC			<0.0001
Non-TNBC	227 (88.0)	25 (55.6)	
TNBC	31 (12.0)	19 (42.2)	
Unknown	0	1 (<1)	
Grade			0.001
I	26 (10.1)	5 (11.1)	
II	145 (56.4)	13 (28.9)	
III	86 (33.5)	27 (60.0)	
Unknown	3(<1)	0	
Recurrence			0.048
No	230 (89.2)	35 (77.8)	
Yes	28 (10.8)	10 (22.2)	
Unknown	2(<1)	0	

Table 9: Univariate Analysis of Patient and Tumor Characteristic in Breast Cancer Patients with and without Tumor Associated Neutrophils.

Tumors with high TAN were compared to tumors with low/negative TAN for their relationship to clinicopathological factors in breast cancer. The p-value was calculated by Fisher's exact test, unknowns were excluded from the analysis.

Factor	HR	Se	P	95% CI	
Stage					
0/I	referent				
II	3.1	1.5	0.02	1.2	8.2
III	14.9	8.0	<0.0001	5.2	42.6
High TAN	2.4	1.0	0.036	1.1	5.5

Table 10: Multivariate Cox Proportional Hazards Analysis of Clinicopathologic Variables' Influence on Breast Cancer Recurrence-free Survival.

Multivariate analysis was performed using the whole cohort (n=305) on all clinicopathological except for ER, PR and tumor grade since those variables were highly associated with TAN in univaritate analysis.

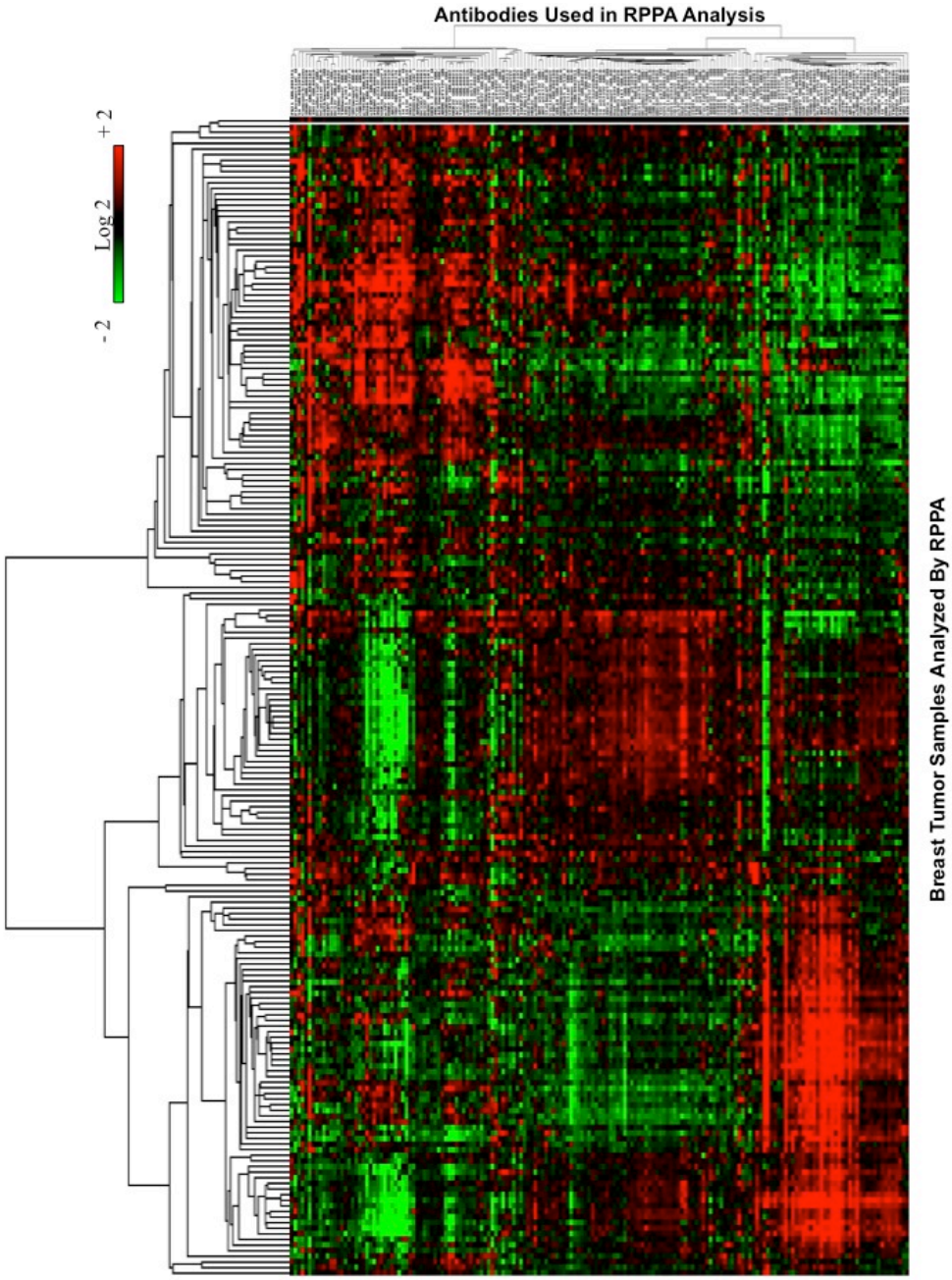


Figure 41: Hierarchical Clustering of RPPA data.

RPPA analysis was performed on 276 breast tumor lysates. Hierarchical clustering was performed on the log_2-median centered values by Pearson correlation (centered) using the Cluster 3.0 software (Eisen Lab) and visualized as a heatmap using treeview (Eisen Lab). Tumors were segregated into three clusters, however these clusters did not correlate with ER, PR, or HER status and did not segregate NE positive and negative tumors. A possible explanation is the neo-adjuvant treatment of these patients.

	Mean (RPPA values)		
	TAN +	TAN -	p-value
	n=85	121	
p90RSK_pT359C_GBL0002019	0.428	0.281	5.43E-16
Rb_pS807V_GBL0001962	0.018	0.014	1.58E-06
HSP27mouseC_GBL0002158	0.272	0.313	1.69E-05
p90RSK_pT359C_GBL0002061	0.416	0.301	1.58E-05
IRS1_p307_GBL0001860	0.0156	0.0176	0.001
EGFR_pY992V_GBL0001791	0.409	0.448	0.002
BIDC_GBL0001878	0.458	0.439	0.005
IGFRbC_GBL0002183	0.181	0.196	0.04

Table 11: Statistically Significant Events Correlating with TAN-Positivity in RPPA Analysis.

Relative abundance of critical nodes in cell signaling pathways was examined using RPPA with 174 validated antibodies. Statistical comparison of TAN-positive and TAN-negative cohorts was performed using the Mann-Whitney *U*-test. The mean of the normalized, linear RPPA values are reported for each group.

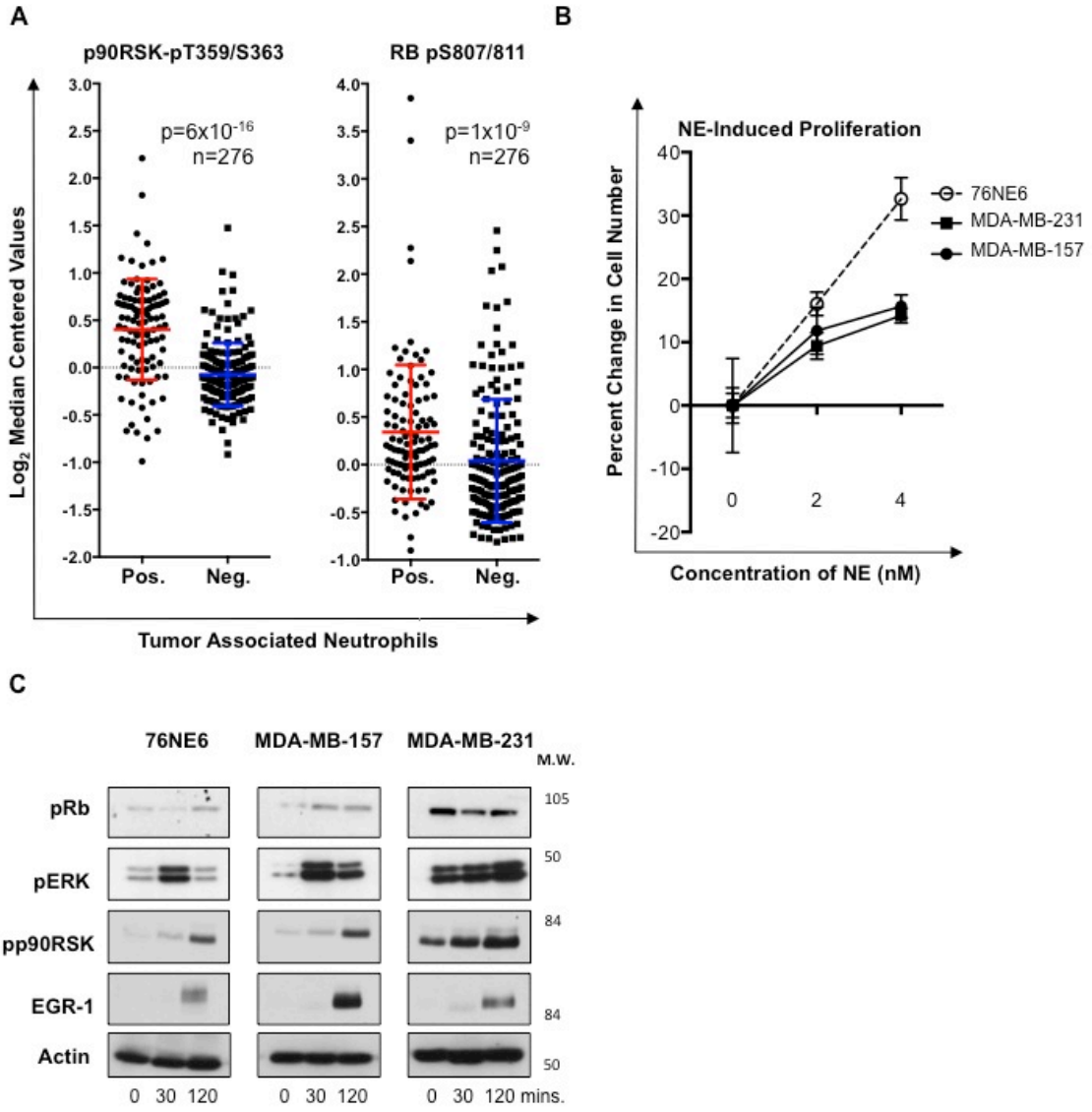


Figure 42: NE-Positive TAN Correlate with the Phosphorylation of ERK-Effector p90RSK and Rb in Breast Tumors.

(A) Relative abundance of pp90RSK T359/S363 and pRb S807/811, according to RPPA analysis. Values represent log_2-median centered values. The p-value was calculated by Mann-Whitney *U*-Test. (B) Change in cell number determined by MTT assay for 76NE6, MDA-MB-231, and MDA-MB-157 treated with 0, 2, 4 nM NE for 48 hours (C) 76NE6, MDA-MB-231, and MDA-MB-157 cell were treated with 10μM NE and subjected to western blot for pRb, pERK, pp90RSK, and EGR-1. Actin, loading control.

NE Knockout Reduces Tumor Growth and Proliferation in the C3(1)TAg Model of TNBC

Genetically engineered mouse models can recapitulate the progression of human breast cancer. Many molecular, histological, and genetic similarities exist, however mouse models fail to completely reproduce the molecular heterogeneity of human breast tumors (779-781). Based on the relatively high concentration of NE in NE-positive TAN observed in human TNBC patients, a mouse model of TNBC was considered ideal to test the hypothesis that NE plays a role in breast tumorigenesis. Relatively few mouse models of TNBC exist, due in part to the use of hormone responsive promoters to target oncogenes to the mammary epithelium.

The C3(1)TAg mouse model has been shown to give rise to TNBC and is molecularly similar to basal-like breast cancer in humans (778, 779, 782, 783). In this model, the expression of SV40 large tumor antigen is directed to the mammary gland by the promoter of rat prostatic binding protein C3 (1) (Pbpc3). This results in the downregulation of p53 and Rb tumor suppressor pathways in the mammary epithelium. Atypia of the mammary ductal epithelium develops at about eight weeks of age, which progress to mammary intraepithelial neoplasia (resembling DCIS) at about twelve weeks of age. Invasive carcinomas develop at about sixteen weeks of age in one-hundred-percent of female mice (male mice get prostate cancer). Lung metastasis develop in ten to fifteen-percent of tumor bearing mice. C3(1)TAg mice are a useful and predictable model of TNBC development (778, 782).

C3(1)TAg mice were crossed with the previously established NE knockout mice (500). C3(1)TAg x NE+/+ and C3(1)TAg x NE-/- cohorts were followed for tumor development for approximately 6 months, at which point all mice had developed tumors (Figure 43A). No statistically significant difference was found in tumor incidence between the C3(1)TAg x NE+/+ and C3(1)TAg x NE-/- cohorts (Figure 43A).

Following tumor initiation, growth was followed until the tumor exceeded the maximal allowable size of 1.5 cm along the greatest tumor axis (Figure 43B). Doubling time was calculated by application of an exponential growth model. C3(1)TAg x NE-/- tumors demonstrated a significantly slower tumor growth rate compared to C3(1)TAg x NE+/+ mice (Figure 43C). To determine if the difference in tumor growth rate was due to altered proliferation, tumors were subjected to immunohistochemical analysis of BrdU

incorporation (mice were injected with 100 mg BrdU/kg body weight two hours prior to sacrifice) (Figure 44A). Quantification reveals significantly less proliferation in C3(1)TAg x NE-/- compared to C3(1)TAg x NE+/+ tumors (Figure 44B). To determine if differences in apoptotic cell death contribute to the observed difference in tumor growth between genotypes, the tumors were subjected to immunohistochemical analysis for cleaved caspase 3 (Figure 45A). No statistically significant difference in the number of cleaved caspase 3 positive cells was observed between genotypes following quantification (Figure 45B).

The mammary glands of C3(1)TAg x NE-/- and C3(1)TAg x NE+/+ genotype mice sacrificed at two months of age were subjected to immunohistochemical analysis of BrdU incorporation (Figure 46A). No statistically significant difference in the percentage of BrdU positive cells was observed (Figure 46B). Immunohistochemical analysis of BrdU incorporation was also preformed in the contralateral mammary gland of tumor bearing mice (Figure 46C). Quantification reveals significantly lower levels of proliferation in the contralateral mammary glands of C3(1)TAg x NE-/- compared to C3(1)TAg x NE+/+ (Figure 46D). These results suggest that NE activity may be an important factor in the growth of pre-invasive lesion in the mammary gland.

NE is a component of the inflammatory response. Deregulated NE activity is known to promote inflammatory gene expression. Deregulated inflammatory signalling is a critical driver of tumor proliferation, therefore we examined the expression of inflammatory gene expression in C3(1)TAg x NE-/- and C3(1)TAg x NE+/+ tumors (Figure 47). Proliferation-associated genes, Melk and Mki67, were significantly downregulated in C3(1)TAg x NE-/- compared to C3(1)TAg x NE+/+ when evaluated by quantitative RT-PCR (Figure 47). However, evaluation of inflammatory-associated genes, Tnf, Il1b, Il6, Csf2, Cxcl1, Ccl3, Mmp9, and Ccl2 does not reveal a statistically significant difference between C3(1)TAg x NE-/- and C3(1)TAg x NE+/+ genotypes (Figure 47). Hierarchical clustering of inflammatory gene expression identifies a cluster of C3(1)TAg tumors with relatively high levels of inflammatory gene expression and a cluster of C3(1)TAg tumors with relatively low inflammatory gene expression. However, clustering based on these failed to differentiate tumors based on the NE-/- or NE+/+ genotype (Figure 48). Taken together these results suggest that NE may have a

specific role in promoting breast tumor cell growth, but not in the inflammatory microenvironment.

To assure that decreased proliferation in C3(1)TAg x NE-/- was not due to a decrease in the infiltration of neutrophils, immunohistochemical analysis of Ly6G (Gr-1) was performed (Figure 49A). Quantification of Ly6G positive neutrophils revealed no statistically significant difference between C3(1)TAg x NE-/- and C3(1)TAg x NE+/+ groups (Figure 49B). Macrophages are an abundant inflammatory component of the tumor microenvironment. Immunohistochemical analysis of F4/80 was performed to assess the concentration of macrophages within the tumor stroma where macrophages predominately localized (Figure 49C). Quantification of peritumoral macrophages reveals no statistically significant difference between the C3(1)TAg x NE-/- and C3(1)TAg x NE+/+ genotypes (Figure 49D).

Western blot analysis was preformed on lysates from twelve tumors from both the C3(1)TAg NE+/+ and C3(1)TAg NE-/- genotypes. Several tumors from NE knockout mice demonstrated slightly higher levels of the NE substrate Irs-1, however the levels were not consistently different between genotypes and no correlation was seen with the phosphorylation of Akt (Ser473) or the Akt substrate GSK3β (Ser9), as previously reported in a lung cancer model (542). Phosphorylated Erk (Thr202/Try204), Erk catalyzed phosphorylation of p90RSK (Thr359/Ser363), and the Erk regulated transcription factor Egr-1 demonstrated no consistent difference in expression between the C3(1)TAg NE+/+ and C3(1)TAg NE-/- tumors. Tlr4, an extracellular receptor activated by NE, was consistently expressed in all samples independent of tumor genotype. The NF-κB pathway inhibitor IκB was not differentially expressed in C3(1)TAg NE+/+ and C3(1)TAg NE-/- tumors. SV40 large T-antigen was expressed at equal levels in both tumor genotypes suggesting that the absence of NE did not compromise the integrity of the C3(1)TAg model (Figure 50).

Taken together the results presented here suggest that NE contributes to tumor growth and proliferation, but is not dependent on upregulation of inflammatory mediators.

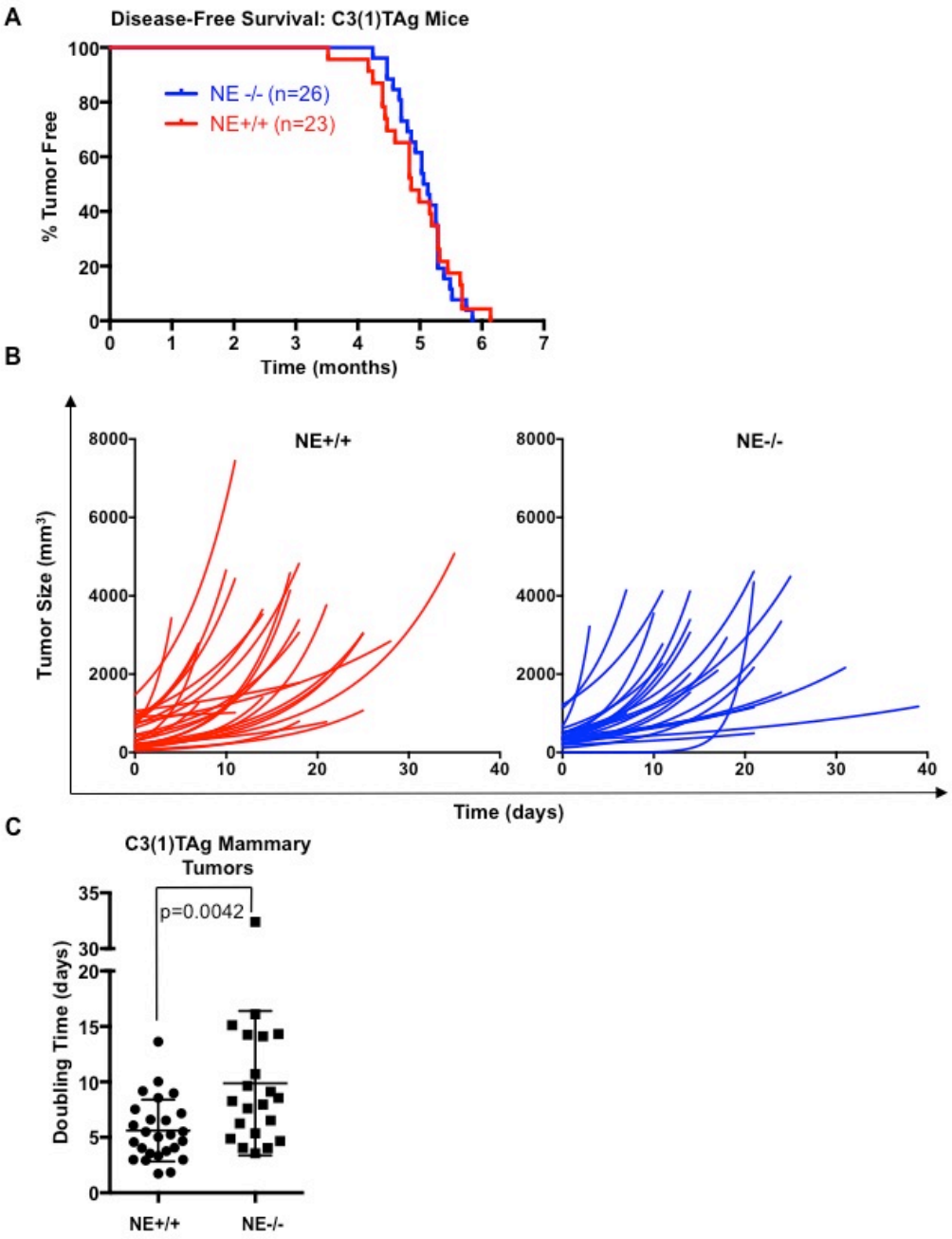


Figure 43: Tumor Incidence and Growth in C3(1)TAg x NE+/+ and C3(1)TAg x NE-/- Genotype Mice.

(A) Kaplan-Meier analysis of tumor incidence in C3(1)TAg NE+/+ and C3(1)TAg NE-/- cohorts. (B) Growth kinetics for individual C3(1)TAg NE+/+ (left panel) and C3(1)TAg NE-/- (right panel). (C) Doubling time was calculated for all C3(1)TAg NE+/+ and C3(1)TAg NE-/- tumors by fitting an exponential growth model.

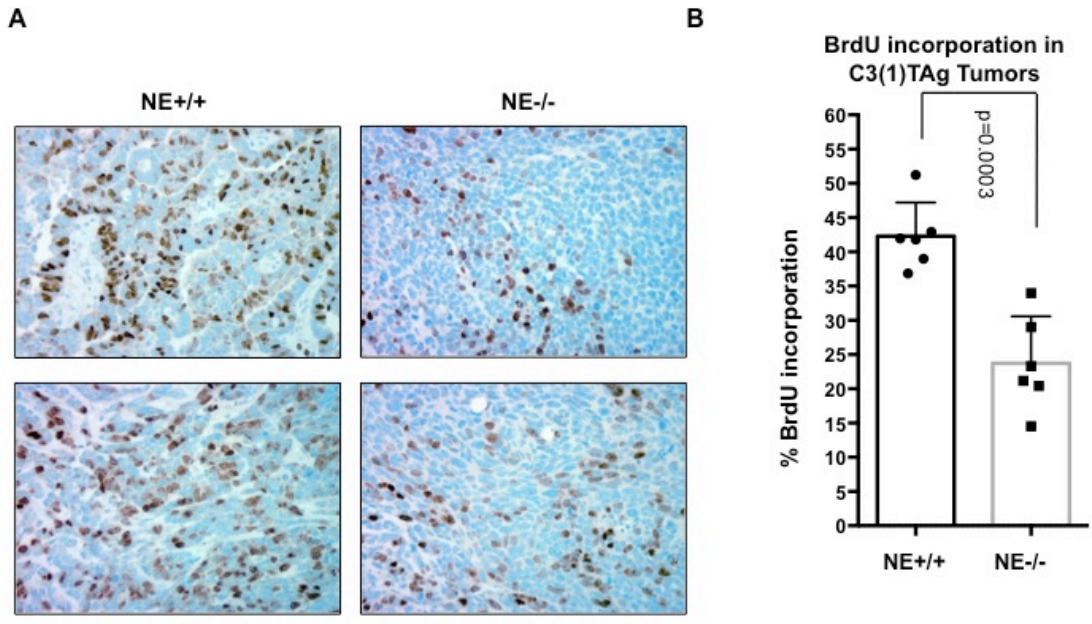


Figure 44: BrdU Incorporation in C3(1)TAg x NE+/+ and C3(1)TAg x NE-/- Genotype Tumors.

(A) C3(1)TAg NE+/+ and C3(1)TAg NE-/- tumors were subjected to immunohistochemical analysis of BrdU incorporation. (B) BrdU positive cells were counted as a percentage of total cells in three representative high magnification fields per tumor, a total of six tumors were examined per group.

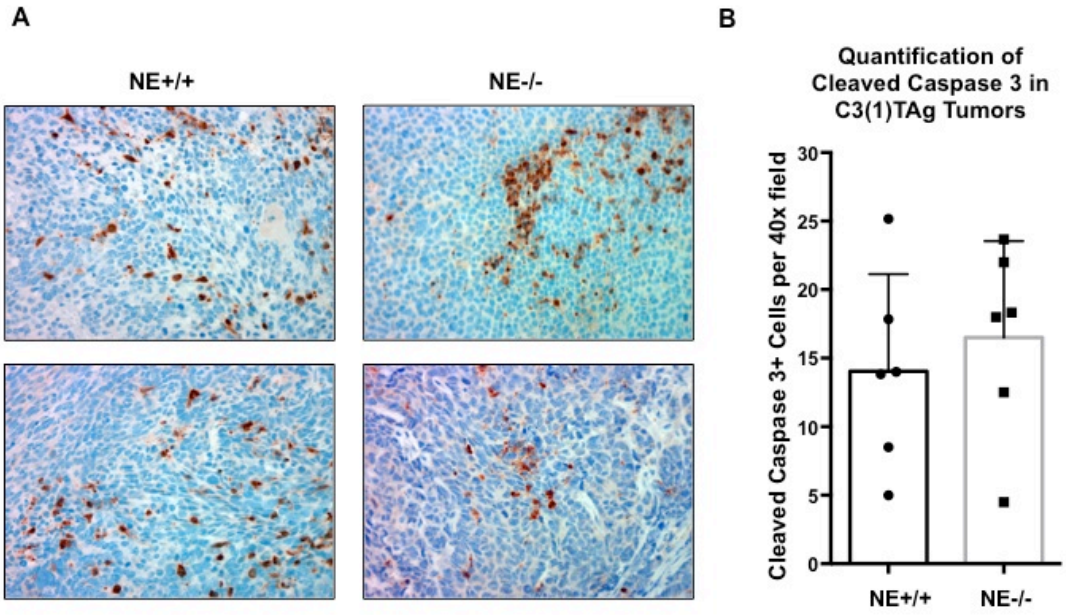


Figure 45: Cleaved Caspase 3 in C3(1)TAg x NE+/+ and C3(1)TAg x NE-/- Genotype Tumors.

(A) C3(1)TAg NE+/+ and C3(1)TAg NE-/- tumors were subjected to immunohistochemical analysis of cleaved caspase 3. (B) Cleaved caspase 3 positive cells were counted in six representative high magnification fields per tumor, a total of six tumors were examined per group

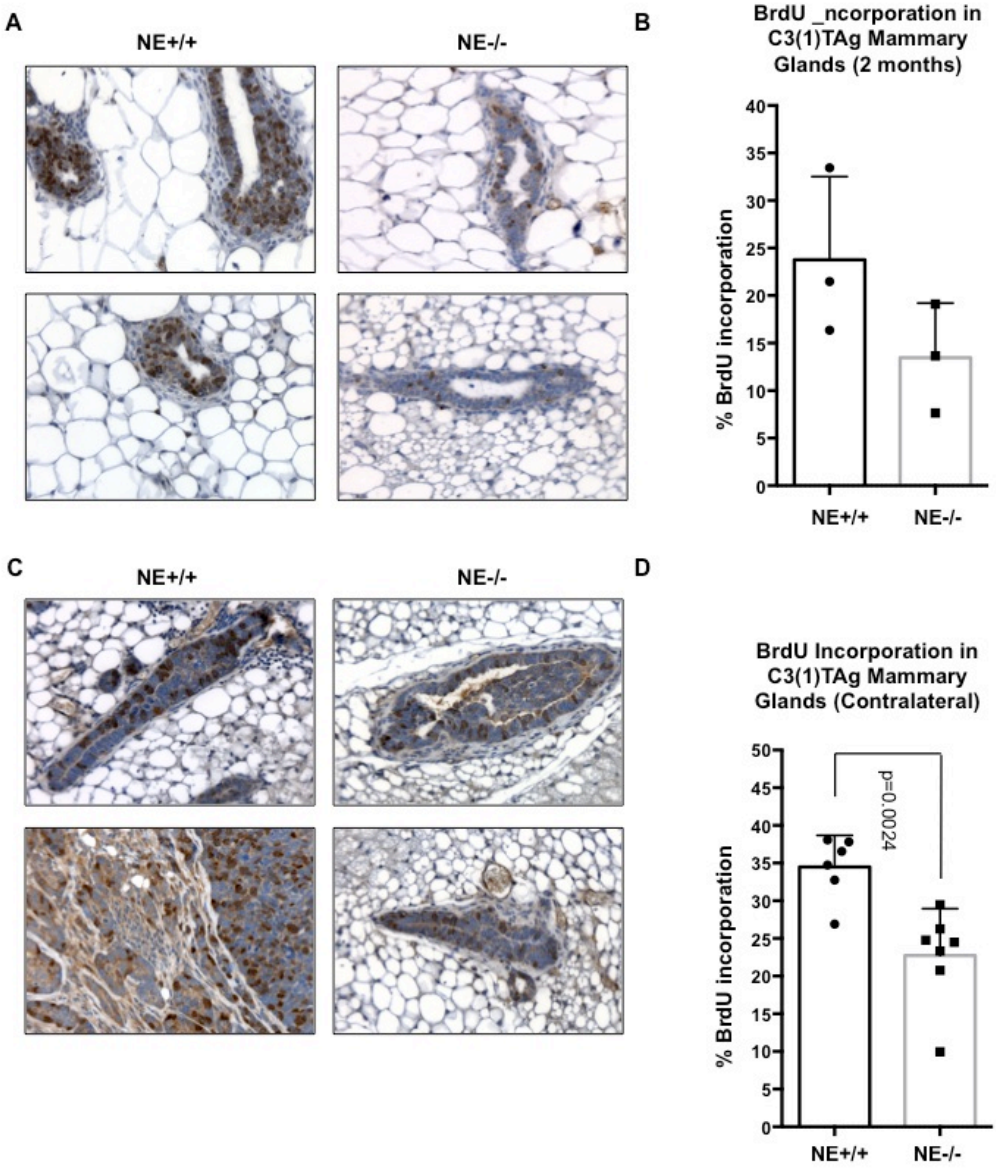


Figure 46: BrdU Incorporation in C3(1)TAg x NE+/+ and C3(1)TAg x NE-/- Genotype Mammary Glands.

(A) C3(1)TAg NE+/+ and C3(1)TAg NE-/- mammary gland from two month old mice were subjected to immunohistochemical analysis of BrdU incorporation. (B) BrdU positive cells were counted as a percentage of total cells in ten representative high magnification fields per section, a total of six sections were examined per group. (C) C3(1)TAg NE+/+ and C3(1)TAg NE-/- mammary gland contralateral to the tumors examined in Figure 39A were subjected to immunohistochemical analysis of BrdU incorporation. (D) BrdU positive cells were counted as a percentage of total cells in ten representative high magnification fields per section, a total of six sections were examined per group.

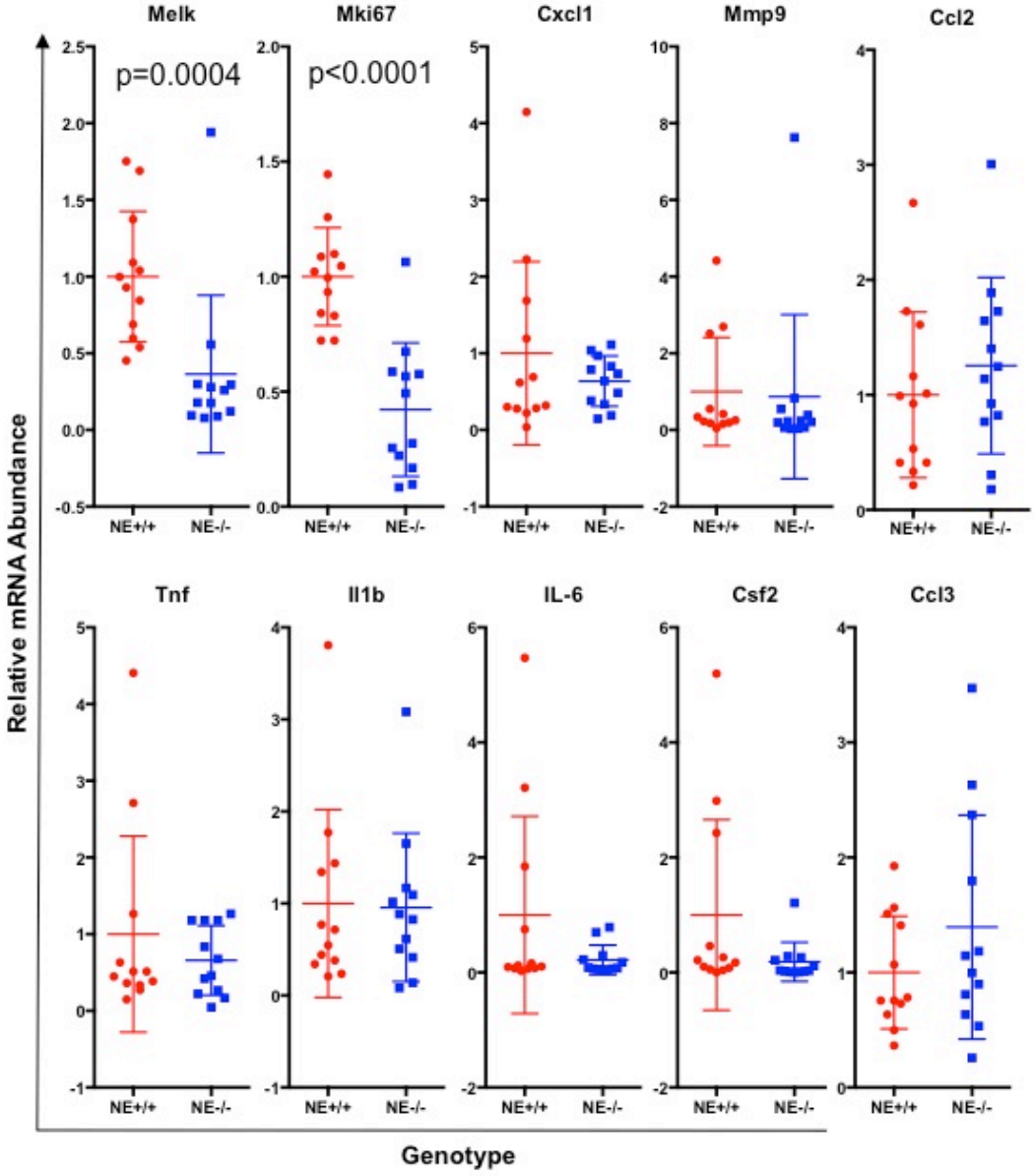


Figure 47: Quantitative PCR of Proliferation and Inflammatory Gene Expression in C3(1)TAg x NE+/+ and C3(1)TAg x NE-/- Genotype Tumors.

(A) Quantitative RT-PCR was used to evaluated the levels of proliferation and inflammation-associated gene expression in C3(1)TAg NE+/+ and C3(1)TAg NE-/- tumors; twelve tumors were examined per group. Values were normalized to Actin expression and are represented relative to mean C3(1)TAg NE+/+ expression.

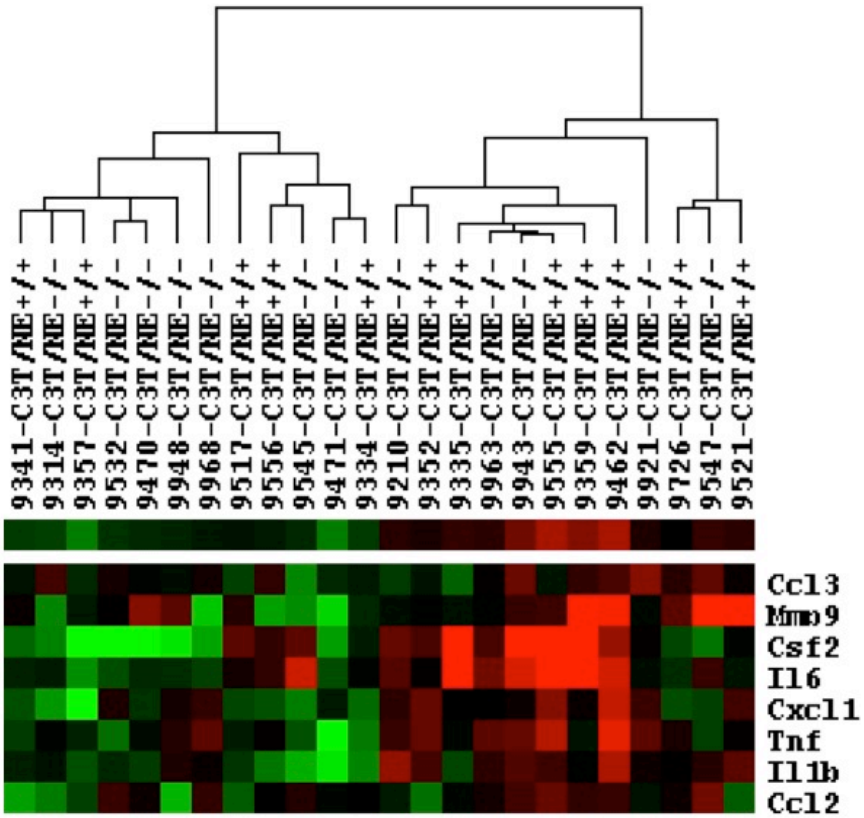


Figure 48: Inflammatory qRT-PCR Gene Signature in C3(1) TAg Tumors

Quantitative RT-PCR was used to evaluated the levels of inflammation associated gene expression in C3(1)TAg NE+/+ and C3(1)TAg NE-/- tumors (some presented in Figure 47). Hierarchical clustering was performed on the $\log_2$-median centered values by Pearson correlation (centered) using the Cluster 3.0 software (Eisen Lab) and visualized as a heatmap using treeview (Eisen Lab).

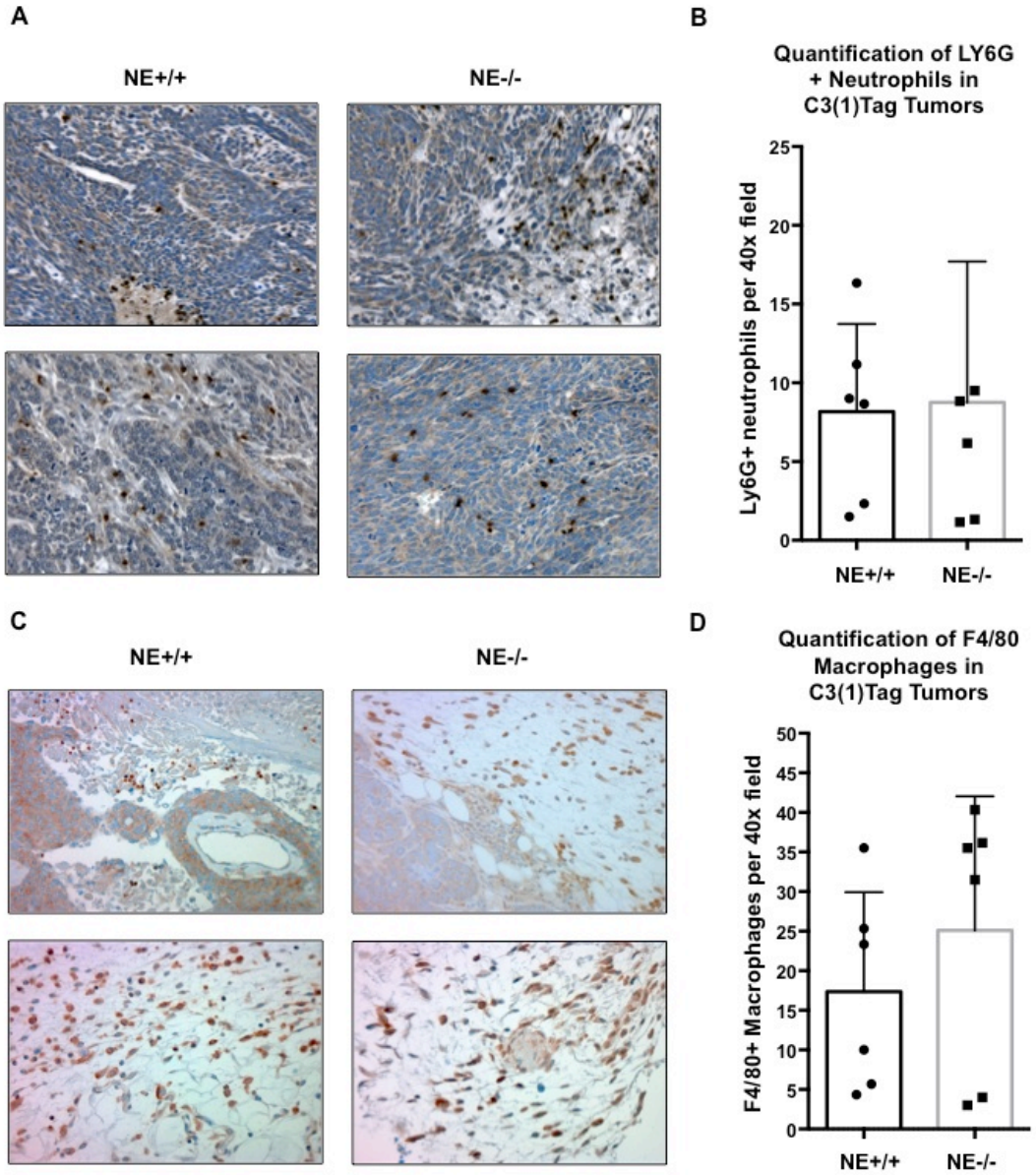


Figure 49: Neutrophil and Macrophage Infiltration in C3(1)TAg x NE+/+ and C3(1)TAg x NE-/- Genotype Tumors.

(A) C3(1)TAg NE+/+ and C3(1)TAg NE-/- tumors were subjected to immunohistochemical analysis of Ly6G a specific marker of neutrophils. (B) Ly6G positive cells were counted in ten representative high magnification fields per tumor, a total of six tumors were examined per group. (C) C3(1)TAg NE+/+ and C3(1)TAg NE-/- tumors were subjected to immunohistochemical analysis of F4/80, a specific marker of macrophages. (D) F4/80 positive cells were counted in ten representative high magnification fields per tumor, a total of six tumors were examined per group. Macrophages accumulate in the peritumoral stroma, therefore representative fields were choose directly adjacent to the tumor epithelium.

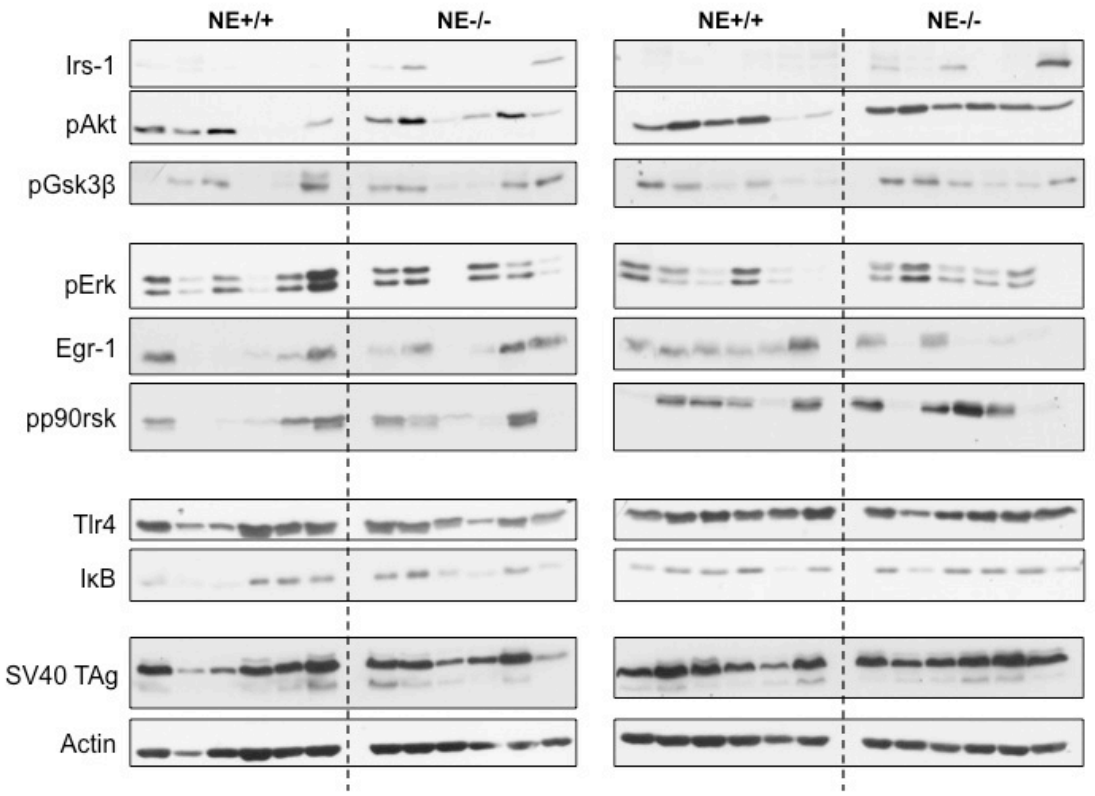


Figure 50: Western Blot Analysis of Signaling Pathways Implicated in NE-Induced Proliferation in C3(1)TAg x NE+/+ and C3(1)TAg x NE-/- Genotype Tumors.

Whole tumor lysates from 12 C3(1)TAg NE+/+ and 12 C3(1)TAg NE-/- genotype mice were subjected to western blot analysis for Irs-1, pAkt (Ser473), pGSK3β (Ser9), pERK (Thr202/Try204), Egr-1, p90RSK (Thr359/Ser363), Tlr4, IκB, SV40 large T-antigen. Actin, loading control.

DISCUSSION

NE expression is restricted to the myeloid lineage. In normal blood, NE immunohistochemistry strongly stains neutrophils and only very weakly stains a subpopulation of monocytes. NE immunohistochemistry also stains neutrophil precursors in the bone marrow and can be used to identify acute myeloid leukemia cells (784). Tumor-promoting myeloid derived suppressor cells, a heterogeneous population of undifferentiated granulocytes often observed in the tumor microenvironment, have been reported to express NE at the mRNA level (785) and may be detected by NE immunohistochemistry performed on tumor sections

Downregulation of elafin in breast tumors suggests increased sensitivity to the growth promoting effect of NE. NE is largely contributed by TAN within the tumor microenvironment (616). The ability of tumor cells to recruit and manipulate nonmalignant cell types, including fibroblasts, endothelial cells, and leukocytes, governs their malignant growth potential (786). Immunohistochemical analysis of NE in a breast tumor specimens revealed that high levels of NE-expressing TAN (Figure 40A). High levels of NE-expressing TAN were prognostic of poor RFS, high tumor grade, and a TNBC phenotype (Figure 40B). These results are consistent with previous studies that found a correlation between high levels of NE measured by ELISA and poor patient survival (12, 599-601). The expression of the endogenous NE inhibitor elafin was also evaluated by immunohistochemistry in this patient cohort. Using the cutoffs established in Chapter Two (Figure 10) elafin expression was found to be downregulated in the majority of invasive breast tumors, validating these results (Figure 39). Therefore, deregulated NE-activity in breast tumors is likely multifactorial, resulting from both the downregulation of endogenous protease inhibitors and the increased concentration of NE secreted by TAN during tumor progression.

RPPA analysis of cancer cell signaling pathways revealed the enrichment of ERK catalyzed phosphorylation of p90RSK at T359/S363 (787) and phosphorylation of Rb at S807/811 in tumors with TAN (NE-positive) compared to tumors without TAN (NE-negative) (Figure 42A). In Chapter two, TLR4-dependent ERK activation and proliferation was observed following the addition of exogenous NE to G0-arrested HMECs. Treatment of breast cancer cell lines and immortalized HMECs with NE is

capable of inducing phosphorylation of p90RSK at T359/S363 and phosphorylation of Rb at S807/811 (Figure 42C), indicating that NE is a TAN-secreted factor capable of enhancing the phosphorylation of these proteins. TAN secrete an array of growth factors, proteases, cytokines, and ROS into the tumor microenvironment

In our analysis, we used a cut-off of 15 TAN per high-magnification field, which is higher than the cut-off used to interrogate the correlation with cell signaling pathways by RPPA (5 TAN per high-power field). Chronic inflammation is known to be an important contributing factor to breast cancer recurrence (367). Use of the higher cut-off to identify the prognostic significance of NE-expressing TAN may identify a group of highly inflammatory tumors. NE-induced TLR4 activation has been shown to induce the expression of pro-inflammatory cytokines/chemokines (590, 737) and may have a role in amplifying the inflammatory response within this subset of breast tumors.

NE-positive TAN were shown to be enriched in TNBC and high-grade tumors (Table 9). NE levels (measure by ELISA) have previously been shown to correlate with ER and PR negative status (12). TNBC highly expresses chemokines critical to the recruitment of TAN, especially IL-8, which may account for the increased number of TAN in these tumors (773-776).

The C3(1)TAg mouse model has been shown to give rise to TNBC tumors that are molecularly similar to basal-like breast cancer in humans (778, 779, 782, 783). Therefore we utilized the C3(1)Tag model of TNBC to test the mitogenic role of NE *in vivo*. No direct homolog of elafin exists within the mouse genome (639), therefore the contribution of elafin downregulation to breast tumorigenesis could not be directly tested *in vivo*. NE knockout were previously generated to study the role of NE in immunity and inflammation (500). NE knockout was shown to limit tumor growth and progression in the *loxP*-Stop-*loxP* K-ras^{G12D} mouse model of lung cancer (542). We found that NE knockout does not slow the kinetics of tumor progression in the C3(1)TAg model (Figure 43A), however NE knockout does reduce tumor growth (Figure 38C) and proliferation (Figure 39A,B).

C3(1)TAg tumors in NE knockout mice demonstrated significantly reduced tumor growth (Figure 43) and proliferation (Figure 44) compared to NE expressing controls. No difference in the level of TAN (Figure 49 A, B), tumor-associated macrophages (Figure S49 C, D), or inflammatory gene expression (Figure 47) was observed between

C3(1)TAg-induced tumors in NE+/+ and NE-/- genotype mice. In this model system, NE has a specific role in the proliferation of tumor cells, but does not appear to alter inflammatory signaling or the recruitment of leukocytes. A study comparing NE knockout and NE/PR3 dual knockout mice found that the ability of NE to cleave the anti-inflammatory factor progranulin and enhance the inflammatory response could be compensated for by PR3 (538). In humans, elafin inhibits both NE and PR3 (13), therefore downregulation of elafin may more broadly sensitize tumors to changes in inflammatory signaling.

The data presented here and elsewhere (508, 542) demonstrate a role of deregulated NE activity in tumor growth and progression, suggesting that NE is an important therapeutic target in cancer

Chapter 5: General Discussion

Elafin is Downregulated During Breast Cancer Progression

Inducible and Systemic Protease Inhibitors

Elafin is an endogenous inhibitor of the serine proteases NE and PR3. Epithelial cells are known to express high levels of elafin in response to pro-inflammatory cytokines (i.e lL-1β and TNF-α) (13, 647). The p38 MAPK, c-JUN, and NF-κB pathways are essential to inducible elafin expression (646, 648, 649). In contrast, systemic serine protease inhibitors, such as α1-antitrypsin and α1-antichymotrypsin, are constitutively expressed by the liver and diffuse into tissues from the circulatory system. Systemic protease inhibitors provide essential baseline control of NE activity; however, they are insufficient to control the high concentrations of neutrophil-secreted NE at sites of inflammation. In this context, elafin is an essential barrier against the deleterious effects of excessive NE activity and necessary for the normal resolution of inflammation (645, 646).

Elafin Expression in Human Tissue Under Normal and Disease Conditions

The normal epidermis does not express elafin (647), however inflammation associated with the hyperproliferative disease psoriasis or experimental wounding was found to enhance elafin expression (650-653). Elafin expression can be detected in bronchial secretions and is hypothesized to be a biomarker of inflammatory lung disease (788, 789). Elafin may also be a biomarker of autoimmune graft versus host disease based on the expression of elafin in the skin rash characteristic of the disease (790). In the endometrium, elafin is highly upregulated during menstruation, suggesting that elafin has a role in physiological tissue remodeling (655). Constitutive elafin expression has been observed at mucosal surfaces, including the tongue, tonsils, gingiva, epiglottis, esophagus, vagina, and pharynx (647). In addition to its protease inhibitory capacity, elafin also has anti-microbial and immune functions, which likely underlies its constitutive expression at mucosal surfaces. Immunohistochemical analysis of elafin expression was performed on several normal human tissues, in

parallel with specimens of normal breast tissue, DCIS, and invasive breast presented in figure 10. This analysis revealed that elafin was expressed by the normal endometrium, kidney, spleen, colon, and mammary gland, but not the prostate (Figure 51).

Inadequate expression or absence of elafin has been observed in disease states characterized by chronic or excessive inflammation, including acute respiratory distress syndrome (14), inflammatory bowel disease (15), and acute lung injury (16). These studies suggest that deregulated NE plays an important role in the pathogenesis of inflammatory disease. NE has been shown to participate in tissue destruction and fibrosis associated with several inflammatory diseases. In COPD and emphysema, NE is viewed as a critical therapeutic target in preventing tissue destruction in inflammatory lung diseases(791).

The tumor microenvironment is characterized by extensive recruitment of inflammatory cells and altered production of inflammatory mediators. The inflammatory components of the tumor microenvironment are indispensible to tumor progression(9). Several studies suggest that loss of elafin-mediated control of NE-activity is a feature of malignant growth. Elafin is downregulated in poorly differentiated squamous cell carcinomas of the skin, head/neck, and esophagus compared to well-differentiated tumors (21, 22). In the majority of melanoma and breast cancer cell lines elafin is downregulated compared to normal HMECs and melanocytes (23, 26). These studies served as the rationale to examine elafin expression in human tumors

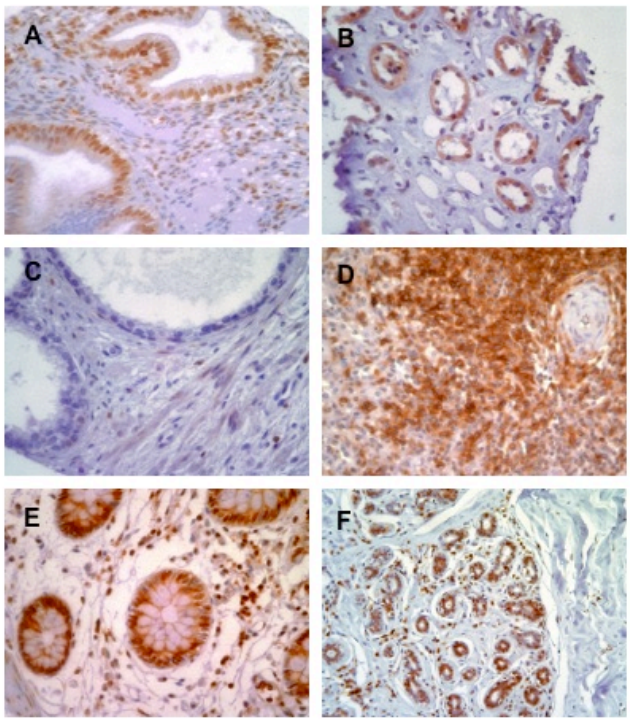


Figure 51: Immunohistochemical Analaysis of Elafin in Normal Human Tissues.

Elafin expression was evaluated by immunohistochemistry in the normal endometrium (A), kidney (B), prostate (C), spleen (D), colon (E), and mammary gland (F).

Immunohistochemical Analysis of Elafin Expression During Breast and Ovarian Cancer Progression

As a first step in establishing a role for elafin in tumorigenesis, immunohistochemical analysis of elafin was performed on normal breast tissue from reduction mammoplasty, DCIS, and invasive breast carcinoma specimens (701). This analysis revealed that elafin is highly expressed in the normal mammary epithelium, but downregulated in the majority of invasive tumors and a subset of DCIS (Figure 10). To determine if elafin downregulation is a generalizable event during tumorigenesis, elafin immunohistochemical analysis was performed on normal fallopian tube, ovarian cystadenomas, borderline tumors, and invasive ovarian carcinoma specimens (792). The majority of ovarian cystadenomas and borderline tumors examined maintained elafin expression at levels similar to the normal epithelium, however elafin expression was downregulated in the majority of invasive ovarian tumors (Figure 12).

Elafin loss in the majority of invasive breast tumors compared to pre-invasive tumors suggests that elafin is specifically switched off as tumors achieve a malignant phenotype. This expression pattern suggests that elafin has tumor suppressive properties early during breast cancer development. Maspin, a member of the serpin family of serine protease inhibitors, was previously reported to have tumor suppressive properties in breast cancer (741, 746, 747). Similar to elafin, maspin expression was originally identified as highly expressed in HMECs, but downregulated in breast tumor cell lines (740, 741). Immunohistochemical studies observed mapsin downregulation in melanoma, breast, prostate, and gastric cancers (742-745). Continued research is necessary to determine if elafin has a bona fide tumor suppressive role in breast tumorigenesis similar to maspin.

Future Direction: Evaluating Elafin as a Prognostic Biomarker in DCIS

In this study elafin expression was not prognostic of survival in breast or ovarian cancer patients (Figure 11 and 13). However, given the preponderant loss of elafin in invasive compared to pre-invasive tumors, elafin downregulation may identify early neoplasias likely to progress to invasive carcinoma. We were unable to evaluate the prognostic significance of elafin in pre-invasive lesions using the patient cohorts presented due to insufficient statistical power and inadequate follow-up. Elafin

downregulation as a prognostic marker of progression in pre-invasive breast DCIS is a particularly interesting hypothesis to be tested if an adequate patient cohort becomes available.

Over the last several decades, increased mammographic screening has contemporaneously led to an increased frequency of DCIS diagnosis. The risk of a subsequent invasive tumor event, following diagnosis of DCIS, is not associated with patient age, race, menopausal status, or a family history of breast cancer. Detection of DCIS by palpation rather than mammography is associated with an increased risk of developing invasive breast cancer. Large tumor size (greater than 10 mm), positive margins, high/intermediate nuclear grade, and the extent or type (comedo) of necrosis are also associated with an increased risk of developing invasive breast cancer. There are currently no reliable molecular biomarkers capable of identifying women diagnosed with DCIS at high risk of developing subsequent invasive carcinoma. Such a prognostic marker could be utilized to better stratify DCIS patients into a cohort requiring extensive therapy and a cohort better served by limited intervention (793).

Following lumpectomy alone approximately 8% of women diagnosed with DCIS will develop ipsilateral invasive breast cancer within 5-years. The addition of radiation therapy reduces the rate of a subsequent invasive event to approximately 3% (794). The low rate of progression limits the ability to develop a sufficiently powered cohort of DCIS patients for the identification of prognostic markers. Very few studies have overcome this barrier to the study of prognostic markers in DCIS. A recently published exception examined a cohort of 1162 women diagnosed (between 1983 and 1994) with DCIS and treated by lumpectomy alone. Specimens from these patients were subjected to immunohistochemical analysis for ER, PR, Ki67, p53, p16, HER2, and COX-2. This study reveals differences in the prognostic factors that are associated with DCIS recurrence and progression to invasive disease. High Ki67, p16, and COX-2 were shown to be associated (independently and together) with increased risk of invasive breast cancer (793).

Elafin may have prognostic significance in DCIS patients that could be evaluated through by immunohistochemical evaluation of elafin downregulation in a large cohort of DCIS patients with sufficient follow-up.

Future Direction: Evaluating the Impact of Polymorphism in the PI3 (Elafin) and ELA2 (NE) Genes on Breast Cancer Incidence

The majority of hereditary breast cancer risk is dependent on the combinatorial effect of several moderate and low penetrance gene variants. Polymorphisms in the PI3 and ELA2 genes have never been examined in a cohort of breast cancer patients versus controls. The hypothesis that an association exists between polymorphisms in these genes and breast cancer incidence could be tested to evaluate an etiological role for deregulated NE activity in breast cancer

Variants in the PI3 gene encoding elafin are associated with the incidence of acute respiratory distress syndrome (ARDS), an disease of excessive lung inflammation. Comparing 449 ARDS patients with 1031 controls a significant association between ARDS and rs2664581 (resulting in an amino acid substitution, elafin T34P). The rs2664581 polymorphism in PI3 was associated with a lower serum concentration of elafin (666).

Polymorphisms in the promoter region of the ELA2 gene encoding NE are associated with lung cancer incidence. Comparing 348 lung cancer patients and 299 controls identified an association between lung cancer and two polymorphisms in the ELA2 promoter -903T/G and -741G/A. A genotype of -903TT was associated with a 2.3 fold greater risk of developing lung cancer, while the -741GG genotype was associated with a 1.4 fold higher risk. Luciferase reporter analysis of both polymorphisms found that they substantially increase ELA2 promoter activity (604). A subsequent study confirmed this association and identified additional polymorphisms in the ELA2 promoter, including -832G/T and -789C/T, that are associated with lung cancer development(795). Another study found no association between polymorphism in the ELA2 gene/promoter and either lung cancer or COPD (796). Polymorphisms in the ELA2 have also been identified as associated with coronary heart disease (797).

A Novel Role for Elafin in Growth Control by Opposing Deregulated NE-Activity

Conventionally Understood Role of Elafin in Inflammatory Disease

Mouse models of elafin overexpression are protected from tissue destruction associated with experimental colitis (667), resistant to acute lung injury (20), demonstrate reduced pulmonary hypertension following chronic hypoxia (19), and

improved heart function after viral myocarditis (18) or myocardial infarction (17). The protease inhibitory capacity of elafin is essential to its anti-inflammatory role in mouse models of emphysema (636). Elafin also has anti-inflammatory properties independent of its anti-protease activity. Elafin directly reduces NF-κB activation in monocytes exposed to LPS through a protease-independent effect on the ubiquitination of NF-κB pathway inhibitor IκB (673). Inducible elafin expression is critical to attenuation of inflammation, prevention of tissue destruction, and preservation of organ function.

Inflammation in the Normal Mammary Gland and Tumorigenesis

Inflammatory signaling networks and cell types play essential roles in the growth, development, and function of the mammary gland. The NF-κB pathway is essential to lobuloaveolar proliferation and differentiation (232). Csf-1 knockout mice are deficient in macrophages and demonstrate impaired branching morphogenesis (217). Mmp-2 knockout mice are incapable of invasion at TEBs and Mmp-3 knockout mice are deficient in lateral side branching (186). Cytokine signaling through Stat3 initiates involution of the mammary epithelium, a process characterized by the infiltration of macrophages and neutrophils (244, 798).

Perturbations in inflammatory signaling and the integrity of ECM are involved in mammary tumorigenesis (8). In mouse models, inflammatory cells are recruited to early adenomas and non-invasive mammary intraductal neoplasia (799) and are detected throughout tumor progression (800). The recruitment of leukocytes is essential to early breast tumorigenesis, growth, and progression (344, 386, 398, 801). Sub-clinical, chronic inflammation is a significant risk factor for the development of human breast cancer (127, 128). Examination of normal breast tissue obtained from reduction mammoplasty confirms that neutrophils ($CD15^{+}CD11b^{+}CD49d^{-}$) are abundantly present even in the absence of neoplasia (802). High levels of NE in breast tumors correlates with poor patient outcome and resistance to therapy (12, 599-601). Studies examining the presence of neutrophils within normal breast tissue and during breast cancer progression are limited. However, the available evidence suggests that neutrophils localize to the mammary gland and are present throughout breast tumorigenesis. The role of neutrophils in the normal mammary gland and during breast tumorigenesis is

poorly understood. A role for the NE activity has never been considered in breast tumorigenesis.

A Role for Elafin in Growth Control

Immunohistochemical analysis revealed that elafin was highly expressed in the normal mammary epithelium. Corroborating this result, HMECs were previously found to express high levels of elafin in cell culture experiments (23). Elafin is downregulated during breast tumorigenesis. To understand the role of elafin in the mammary epithelium and the impetus for its downregulation during tumorigenesis we employed HMECs as a model system.

Elafin is highly expressed by growth factor deprived G0 HMECs compared to HMECs proliferating in growth factor containing media or arrested within the cell cycle (Figure 14 and 15). This result suggests a previously unrecognized role for elafin in growth control. Elimination of G1/G0 checkpoint control by downregulation of the Rb tumor suppressor results eliminates the ability of HMECs to enter G0 and upregulate elafin (Figure 18 and 20). C/EBP β sites (24) in the elafin promoter are also necessary for expression of elafin in G0 HMECs (Figure 17). The integrity of the Rb and C/EBP β pathways are likely essential to the constitutive expression of elafin in the normal breast epithelium. Dominant negative C/EBP β is predominately expressed (as a ratio to full-length, activating isoforms) in a large proportion of breast tumors (24). The Rb tumor suppressor is inactivated by directly by mutation and allelic loss and indirectly by alteration in proteins controlling Rb levels and activity(85).

Elafin knockdown HMECs fail to maintain G0-arrest during long-term growth factor deprivation. The elafin protease inhibitory domain is essential to its anti-mitogenic capacity (Figure 21). Elafin knockdown HMECs demonstrate increased sensitivity to the growth promoting effect of exogenous NE (Figure 22). Several published studies have demonstrated increased proliferation following addition of exogenous NE (542, 676, 736). *In vivo* the application of NE to the mouse epidermis results in proliferation, whereas application of elafin can prevent epidermal proliferation (676). Mechanistically, TLR4 dependent ERK activation is essential to the growth promoting effect of NE (Figure 25). Overall, these results suggest that elafin is an essential component of

epithelial growth control as a critical counterbalance against mitogenic effect of NE in normal epithelial cells.

Future Direction: In Vivo Role of Protease Inhibitors in Breast Tumorigenesis

Mice do not have a direct elafin homolog. However, secretory leukocyte peptidase inhibitor (Slpi) shares structural similarity with the elafin WAP domain and has the capacity to inhibit NE. Unlike elafin, Slpi is incapable of inhibiting Pr3 and can inhibit trypsin, chymotrypsin, and cathepsin G.

Experimental evidence from mouse models of inflammatory disease suggests that Slpi is a critical counterbalance against deregulated NE activity. The colonic epithelium of thymic stromal lymphopoetin (Tslp) knockout mice orally administered dextran sodium sulfate (DSS) failed to upregulate Slpi, demonstrated high NE activity, and were unable to recover from colitis compared to controls. Administration of recombinant Slpi or the NE inhibitor sivelestat enhanced recovery from DSS-induced colitis in Tslp knockout mice (803). Slpi knockout mice demonstrate impaired healing following cutaneous wounding. Wounding healing in Slpi knockout mice was characterized by increased inflammation, excessive production of Tgfβ, and high NE activity (804). Progranulin (also known as proepithelin) is a critical growth and regulatory factor in the wound healing response. NE cleaves progranulin into a form that is detrimental to normal wound healing. Addition of full-length progranulin to the wounds of Slpi knockout mice restores the wound healing response (539). Roles for Slpi in inflammation and immunity have also been identified independent of its anti-protease capacity (805, 806).

Studies investigating the role of SLPI in cancer have often arrived at seemingly incompatible conclusions. SLPI has been shown to have tumor promoting (760-763) and tumor suppressive effects (761, 764, 765) in xenograft mouse models. In these experiments, SLPI was overexpressed in tumor-derived cell types prior to ortotopic or sub-cutaneous injection. The pro-tumor effects of SLPI overexpression appear to be independent of its anti-protease activity (760) and highly dependent on the tumor type being examined (761). Anti-tumor effects were exclusively observed in mammary tumor xenografts (761).

A more complete understanding of Slpi-mediated protease inhibition could be obtained by crossing the Slpi knockout mouse with transgenic breast tumor models. This experiment would test the hypothesis that disequilibrium between NE and its inhibitor Slpi promotes breast tumorigenesis and progression *in vivo*. The contribution of inflammatory processes to breast tumorigenesis has previously been examined in the MMTV-PyMT transgenic mouse model. MMTV-PyMT tumors demonstrate abundant infiltration of neutrophils (presumably expressing NE). The progression of MMTV-PyMT tumors has been carefully delineated making it possible to examine the effects of changes in NE activity throughout tumor progression (800). MMTV-PyMT tumors are also highly metastatic, making this transgenic model ideal for the study of deregulated NE in metastatic breast cancer. In chapter 4, the C3(1)TAg model was utilized to understand the role of NE in TNBC. In this model, NE knockout significantly reduced the level of proliferation (Figure 44). Knockout of Slpi in these mice may enhance proliferation in tumors and pre-invasive lesion. C3(1)TAg are not highly metastatic, a major drawback to this model.

Future Direction: The Role of TLR4 in Breast Tumorigenesis

TLRs recognize molecules derived from invading microbes, termed pathogen-associated molecular patterns, and are essential to the innate immune response. TLR4 specifically recognizes lipopolysaccharide (LPS). Activation of TLR4 by LPS requires several auxiliary proteins, including LBP and CD14, and the co-receptor MD-2. Stimulation of TLR4 by LPS results in the intracellular recruitment of adaptor proteins MyD88 and TRIF, which activates of the NF-κB, ERK, and JNK pathways leading to the expression of pro-inflammatory cytokines and interferons (807). Treatment of murine macrophages with NE resulted in the upregulation of cytokines Tnfa, Il1b, Cxcl1, and Il6, while Tlr4 knockout macrophages are incapable of upregulating these cytokines upon NE stimulation (597). In bronchial epithelial cells NE induced IL-8 expression through TLR4 activation (590). Direct proteolysis of TLR4 by NE has been reported (737). However, the NE cleavage sites on TLR4 have not been identified. Mapping the cleavage of TLR4 by mass spectrometry or another biochemical technique is important to the development of a complete understanding of NE-induced TLR4 activation. The effect of NE-mediated cleavage on the recruitment of auxiliary proteins and the

activation of downstream signaling must also be resolved by biochemical analysis. The relevance of NE-mediated cleavage of TLR4 to human breast tumorigenesis is an important question. Antibodies that identify cleaved and uncleaved TLR4 could facilitate detection of truncated forms in tumors (737).

Tumor cells have been shown to express TLR4, however the expression of TLR4 is highest in macrophages, dendritic cells, and other immune cell types within the tumor microenvironment (808, 809). TLR4 activation can result in the activation of iNOS, IL-6, uPA, IL-10, IL-8, TGFβ, and VEGF (809, 810), factors with known roles in tumor growth and progression suggesting that TLR4 has pro-tumorigenic properties. In chronic liver disease, intestinal bacterial infiltrate the liver and result in the activation of TLR4. Activation of TLR4 in the liver is critical to the development and progression of heptacellular carcinoma in mouse models (811). In experimental models of breast and ovarian cancer, downregulation of TLR4 has been shown to reduce the growth and progression of xenograft tumors (812, 813).

Microbial-derived TLR4 ligands are not found at many sites of tumor initiation, including breast and ovarian cancer (808). Endogenous ligands of TLR4 are believed to play an important role in the activation of TLR4 in these tumors. High-mobility group box-1 (HMGB1) has been identified as an endogenous ligand of TLR4 (809). HMGB1 is a nuclear protein that is secreted by necrotic cells (814, 815). NE may be an important instigator of TLR4 activation during inflammatory conditions (590, 597).

TLR4 can also play a role in anti-tumor immunity. In breast cancer patients treated with chemotherapy or radiotherapy, HMGB1 released by dying cells activates TLR4 expressing dendritic cells resulting in an anti-tumor immune response. Patients with a TLR4 loss of function allele relapse earlier following chemotherapy or radiotherapy compared to patients with fully functional TLR4 indicating that initiation of an innate immune response is an indispensible component of cancer therapy (816).

The available evidence suggests that TLR4 activation can dramatically enhance pro-tumorigenic cytokine and growth factor signaling or initiate anti-tumor immunity depending on context and the cell type affected. Mice bearing a point mutation to the third exon of Tlr4 (resulting in a proline to histidine substitution at position 712) have been developed. These mice demonstrated defective Tlr4 activation following LPS stimulation and extreme susceptibility to bacterial sepsis (817). To test the hypothesis

that epithelial Tlr4 promotes tumorigenesis, the mammary epithelium from mice transgenic for MMTV-PyMT and mutant TLR4 can be transplanted into the cleared mammary fat pad of wild type. To test the hypothesis that Tlr4 in the stromal compartment accelerates tumorigenesis the MMTV-PyMT transgenic mammary gland expressing wild type Tlr4 can be transplanted into the cleared mammary fat pad of Tlr4 mutant mice. Adoptive transfer of TLR4 mutant bone marrow into MMTV-PyMT transgenic mice could be used to test the hypothesis that Tlr4 activation leukocytes promotes tumorigenesis. Alternatively, to test the hypothesis that leukocyte Tlr4 is essential to the anti-tumor response these mice could be treated by irradiation or chemotherapy and evaluation of tumor regression.

Conditional TLR4 knockout mice have been recently developed (818) providing an alternative model to specifically eliminate TLR4 expression from the mammary epithelium and specific cell types within the bone marrow or stromal compartments.

Elafin has Tumor Suppressive Properties

Published studies have observed downregulation of elafin in breast tumor derived cell lines compared to HMECs (23, 24). To test the hypothesis that elafin has tumor suppressive properties, elafin was expressed in breast tumor-derived cell lines using an adenoviral vector. Elafin expression caused cell cycle arrest in breast cancer cell lines expressing the Rb tumor suppressor. However, in breast cancer cell lines lacking Rb, elafin expression induces apoptotic cell death (Figure 31). Elafin expression does not affect HMECs, which endogenously express elafin. However, HMECs with a compromised Rb-dependent, G1-checkpoint fail to express endogenous elafin and are sensitive to the adenoviral elafin-induced apoptotic cell death (Figure 30). Overall, the expression of elafin causes cell cycle arrest or apoptosis in breast tumor derived cell lines depending on Rb status, but does not affect normal HMECs with an intact G1 checkpoint Elafin represents a candidate therapeutic capable of specifically targeting tumor cells with disruption of the G1 checkpoint with no toxicity in normally dividing cells.

Since the publication of this work (678), intratumoral injection of adenoviral elafin into MDA-MB-468 xenograft tumors was shown to reduce tumor burden and prolong the survival of mice (25). Elafin re-expression using a tet-inducible system resulted in

apoptotic cell death in melanoma cells, but not normal melanocyte. In melanoma xenografts, expression of elafin significantly reduced tumor size (27).

Future Direction: Identification of the Mechanism by which Elafin Induces Apoptotic Cell Death

The work presented here and elsewhere (25-27), reporting the ability of elafin to induce apoptosis in breast and melanoma cell lines, does not address if the apoptotic effect of elafin is protease dependent or independent. NE expression has previously been characterized in tumor cell lines (25, 617, 735). In experimental models, pharmacological NE inhibitors were able to attenuate the development of skin tumors, reduce growth and metastasis in a lung xenograft model, and inhibit proliferation/chemotaxis of pancreatic cells (603, 606, 607, 609). Knockdown of NE has been shown to decrease migration and growth of breast tumor cell lines (25, 617). In these experiments no effect was observed on cell survival, however MDA-MB-231 cells were the predominately utilized model system. In Figure 34, MDA-MB-231 cells were not sensitive to the apoptotic effect of elafin unless Rb was experimentally downregulated. To address the hypothesis that the apoptotic effect of elafin on breast cancer is protease dependent, previously reported mutations to the protease inhibitor domain of elafin that inactivate its anti-protease activity should be utilized (636). Inducible expression of these mutants elafin M25G and elafin M25K should be evaluated by TUNEL and BrdU incorporation in the panel of cell lines that are sensitive to elafin-induced growth arrest (MCF-7, ZR75-1, and T47D) and apoptosis (MDA-MB-157, MDA-MB-436, and MDA-MB-468).

Future Direction: Therapeutic Reactivation of Elafin in Breast Tumors

In xenograft models, adenovirus was directly injected into tumors (25), while this is an excellent proof of principle it is unlikely to be a viable clinical strategy given the clinical restraints on gene therapy. However, a pharmacologically active compound capable of inducing elafin re-expression in tumor cells could be a clinically translatable.

In melanoma cell lines, normally lacking elafin expression, treatment with a DNA methyltransferase inhibitor induced the expression of elafin. The elafin promoter was not directly methylated in melanoma cells. Demethylation of the FOXA2 promoter resulted in restoration of FOXA2 levels and elafin transcripiton. In these experiments,

elafin knockdown melanoma cells demonstrate reduced sensitivity to apoptotic cell death induced by DNA methyltranferase inhibitor (26). This experiment suggest that elafin reactivation by therapeutics may be an important determinate of cytotoxicity.

Oltipraz is a chemopreventative agent that has been shown to induce C/EBP β DNA binding and activation of gene transcription both *in vivo* and *in vitro* (819-824). Oltipraz has been used in clinical trials as both a chemopreventative agent (825) and in the treatment of patients with liver fibrosis and cirrhosis (821), however the results of these studies are inconclusive. C/EBP β is essential to the expression of elafin in HMECs and deregulation of C/EBP β is responsible for elafin downregulation in breast cancer cell lines (24). Therefore, oltipraz may be capable of enhancing the transcription of elafin in breast tumor cells and may be a promising anti-cancer agent.

The ability of oltipraz to induce elafin expression should be examined, particularly in Rb-negative breast cancer cell lines. To determine if oltipraz can induce apoptotic cell death through elafin upregulation, Rb-negative breast cancer cell lines expressing elafin shRNA can be treated with the oltipraz. If these experiments, prove successful, pre-clinical models combining elafin-inducing oltipraz with traditional chemotherapy or targeted therapies could be tested.

More specific elafin-inducing drugs could be identified using the previously established elafin luciferase reporter system (24). This system consists of a firefly-luciferase promoter constructs containing the 440 bp proximal elafin promoter (pSPL440) and the 440 bp proximal elafin promoter with mutations to C/EBPβ binding sites 4 and 5 (pSPL440m4/5), which are critical to elafin transcription. Breast cancer cell lines stably incorporating these plasmids could be used to screen for drugs that potently induce elafin expression specifically through C/EBPβ. NF-κB-dependent (717) expression of elafin has been described in response to pro-inflammatory cytokines, especially IL-1β and TNF-α (646, 647). Avoiding drugs that induce elafin through this pathway is important given the tumor promoting effect of NF-κB.

Neutrophil Elastase Promotes Breast Cancer Growth *In Vivo*

Conventionally Understood Role of NE in Inflammation and Tumorigenesis

NE is normally sequestered within the azurophilic granules of neutrophils and is essential to the intracellular destruction of pathogens following phagocytosis at sites of infection (500). Activated neutrophils secrete NE into the extracellular environment. Extracellular NE activity is associated with degradation of the ECM and disruption of cell adhesion, which plays a role in tumor invasion and metastasis. NE has also been implicated in the alteration of cytokine and chemokine signaling resulting in increased infiltration and activation of pro-inflammatory cell types. The conventionally understood role of NE in tumor progression emphasizes the importance of NE in cell invasion and metastasis, through ECM degradation and the cleavage of adhesion molecules (610).

Role of NE in Intracellular Signaling

Several groups have demonstrated a role for NE in intracellular signaling. NE activates cell surface receptors, including TLR4 (590), proteinase-activated receptor 2 (PAR2) (521), and epidermal growth factor receptor (EGFR) (681), either directly through proteolysis of the extracellular domain or indirectly through the liberation/activation of latent ligands within the ECM (506). These pathways have never been studied in tumorigenesis and are largely known from examining the role of NE in inflammatory lung disease. Tumor cells demonstrate the capacity to endocytos NE from the extracellular microenvironment. Intracellular NE was shown to enhance phosphoinositide 3-kinase (PI3K) activity following the degradation of insulin receptor substrate-1 (542). NE has also been implicated in cleavage of cyclin E into low-molecular weight isoforms capable of hyperactivating cyclin-dependent kinase 2 and inducing tumor formation in mouse models (541, 624).

Tumor Associated Neutrophils and NE in Tumorigenesis and Progression

Tumor-infiltrating neutrophils (TAN) are recruited by cancer cell-derived chemokines (368) and are prognostic of poor patient outcome in several tumor types (410, 826). Depletion of neutrophils or disruption of neutrophil chemotaxis inhibits tumor growth, angiogenesis, and metastasis in mouse models of tumor progression (415-417). NE knockout in the *loxP*-Stop-*loxP* K-ras^{G12D} mouse model of lung cancer severely

limits tumor growth and proliferation, providing direct *in vivo* evidence of a role for NE in lung tumorigenesis (542). NE-mutant mice ware protected from skin carcinogenesis following ultraviolet irradiation and benzopyrene exposure (609).

The evaluation of NE by immunohistochemistry reveals that high levels of NE-expressing, tumor-associated neutrophils were prognostic of poor recurrence-free survival and correlate with intracellular signaling events. This observation is consistent with several previous studies that measure NE in breast tumors by ELISA, which found a strong correlation between high levels of NE and poor patient outcome (12, 599-601).

Reverse phase protein array (RPPA) analysis of critical signaling pathways revealed a correlation between NE-expressing neutrophils and phosphorylation p90RSK (ERK-dependent) and Rb. In chapter 2, TLR4 dependent ERK activation was found to be essential to the growth promoting effect of NE. Previously published studies have also observed that NE can induce ERK signaling. (681). An enrichment of ERK-specific phosphorylation of p90RSK in NE-positive tumors suggests that this observation is an important downstream effector of TAN capable of inducing ERK signaling and cell proliferation.

NE-positive TAN were associated with TNBC and high-grade tumors. High NE expression (determined by ELISA) was previously shown to negatively correlate with tumor ER and PR status (12). TNBC highly expresses chemokines critical to the recruitment and activation of TAN, including IL-8 and IL-6 (773-776). Based on the high concentration of NE-positive TAN in TNBC we chose to examine the role of NE in a mouse model of TNBC. The C3(1)TAg mouse model has been shown to give rise to TNBC tumors (778, 779, 782, 783). NE knockout does not slow the kinetics of tumor progression in the C3(1)TAg model (Figure 43A), however NE knockout does reduce tumor growth (Figure 38C) and proliferation (Figure 39 A, B).

By extending the role of NE beyond tumor cell invasion, our results argue that the application of therapeutic modalities targeting NE activity could inhibit multiple elements of tumor growth and progression. NE inhibitors are currently under development for the treatment of chronic inflammatory lung diseases. This study positively contributes to the rationale for examination of these inhibitors as anti-cancer agents. Many studies suggest that the recruitment of non-malignant cell types is an essential component of breast tumor progression. Targeting essential crosstalk between the tumor epithelial

compartment and the stromal microenvironment is a promising approach to anti-cancer therapy.

The NE inhibitor sivelestat, was able to reduce the proliferation, motility, and chemotaxis of the pancreatic cancer cell line Capan-1 *in vitro* (603). In a mouse xenograft model of non-small cell lung cancer using the EBC-1 and PC-1 cell lines, sivelestat attenuates proliferation and metastasis (606). Sivelestat also inhibited spontaneous metastasis of EBC-1 xenograft tumors (607). Beige mice are deficient in NE (608). Pharmacological inhibitors of NE, 2,4,6-trinitrochlorobenzene and oxazolone were able to attenuate the development of skin tumors following ultraviolet irradiation (609).

Future Directions: Alternatives to the C3(1)TAg Model to Determine a Role for NE in Metastatic Breast Cancer

C3(1)TAg tumors were poorly metastatic. The lungs of C3(1)TAg tumor bearing mice were inflated with formalin, fixed, paraffin-embedded, and sectioned for immunohistochemical analysis. Following staining with an antibody specific to SV40 large T-Antigen (data not shown) only 4 out of 49 mice had metastatic lesions (two were of the NE+/+ genotype and two of the NE-/- genotype). Interestingly, the NE+/+ mice with metastatic lung lesions had a greater number of foci and larger lesion size than NE-/- mice, however no conclusions could can be drawn about the influence of NE on metastasis in this model. The MMTV-Wnt1 mouse models and the $BRCA^{fl/fl}$p53+/- also generate tumors with a TNBC phenotype, however they are also poorly metastatic (827, 828). To test a role for NE in metastatic breast cancer the NE knockout could be examined in the MMTV-PyMT transgenic model, which is highly metastatic (404, 779). Alternatively, *in vivo* passaging of the C3(1)TAg tumors could be performed to create transplantable mouse tumors with increased metastatic proclivity and then injected into the mammary fat pads of NE+/+ and NE-/-.

Future Directions: Development of Pre-Clinical Models of NE-Inhibition in Breast Cancer.

AZD9668, a pharmacological NE inhibitor, was well tolerated by healthy individuals and was found to possess favorable pharmacokinetics in phase I clinical trials (829). In phase II clinical trials, AZD9668 did not alleviate the symptoms of COPD patients (598, 830) and had limited benefit in patients with bronchiectasis and cystic

fibrosis (831, 832). AZD9668 diminished levels of pro-inflammatory biomarkers in the bloodstream and levels of NE specific elastin catabolites, desmosine and isodesmosine in the urine (831, 832). Collectively, these studies suggest that AZD9668 is not an effective therapy in inflammatory lung disease. However, these studies were relatively short term (less than one month) and the results of future long term clinical trials should be considered.

AZD9668 is capable of inhibiting NE-activity in human patients (831, 832) and patient derived neutrophils (833). Oral administration of AZD9668 to rats or mice prevented NE-induced lung injury and reduced inflammation following exposure to cigarette smoke (833). AZD9668 may be an effective inhibitor of NE in tumor models.

NE knockout reduces tumor growth (Figure 38C) and proliferation (Figure 39A,B) in the C3(1)TAg of TNBC. The MMTV-PyMT breast tumor model has been suggested throughout this discussion as a possible alternative to examine the role of NE in metastatic breast cancer largely based on the abundant infiltration of neutrophils in MMTV-PyMT (800). To testing the efficacy of AZD9668 in breast cancer, C3(1)TAg and MMTV-PyMT transgenic mice could be orally administered AZD9668 following tumor initiation at approximately 4 months, proliferation, absolute growth delay, and metastasis as primary endpoints. As a preventative therapy AZD9668 could be administered beginning at four weeks of age with tumor-free survival as the primary end point.

Chemotherapeutic agents activate tumor fibroblast resulting the production of inflammatory cytokines and chemokines resulting in a robust inflammatory resoponse and the recruitment of neutrophils and macrophages (348, 834). TAN in the tumor microenvironment likely contribute NE, which may have a role in chemoresistance and recurrence based on the results presented within this dissertation. Therefore combination of cytotoxic chemotherapy with AZD9668 may be an effective anti-tumor strategy. This hypothesis could be tested in traditional cell line xenograft models of breast cancer or in patient specimen derived explant models which are more representative of human tumors. In these models, tumor regression, survival, and recurrence could be examined following co-administration of AZD9668 and cytotoxic chemotherapeutics, such as doxorubincin.

Conclusions

The interdependency of tumor cells and the microenvironment in which they evolve (7) provides a strong rationale for the therapeutic targeting of essential crosstalk. The cellular constituents of the tumor microenvironment are not susceptible to the selective pressures driving therapeutic resistance in tumor cells; therefore, therapeutic modalities targeting critical microenvironmental factors may yield durable antitumor responses.

The data presented here suggests that NE is an important downstream effector of TAN capable of inducing ERK signaling and cell proliferation. The absence of elafin in the majority of breast tumor enhances sensitivity to the mitogenic effects of NE. Elafin has tumor suppressive properties *in vitro*. The absence of NE significantly decreased tumor growth and proliferation *in vivo.* A therapeutic approach directed specifically at controlling deregulated NE-activity may therefore be an effective therapy in breast cancer. Effective inhibitors of NE have been developed for the treatment of inflammatory lung disease (11).

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Vita

Joseph Anthony Caruso was born in Fredericksburg Virginia on October 13, 1984 to parents Joseph A Caruso and Rosemary Barra Caruso. Sharing his first name with his father, the son has always gone by his middle name, Tony, to avoid confusion and having his name affixed with a detestable suffix “Joe Jr.” or even worst a prefix “Little Joe.” Tony has a twin sister, Andria Marie Caruso, who is now a medical intern specializing in ear, nose, and throat at the Walter Reed National Military Medical Center in Bethesda Maryland. Clearly, higher education is a priority in the Caruso family. The elder Joseph is a computer engineer at the Naval Surface Warfare Center Dahlgren Division and Rosemary is a distinguished professor of biology and former Dean at the University of Mary Washington. After graduating from Stafford Senior High School in 2003, Tony attended Villanova University to study Biology. He completed a senior honors thesis under the mentorship of Dr. Louise Russo Ph.D. Tony graduated from Villanova University in May of 2007 with a B.S. in Biology. Following graduation, Tony enrolled in the University of Texas Graduate School of Biomedical Sciences to pursue a Ph.D. under the mentorship of Dr. Khandan Keyomarsi Ph.D. On a personal note, Tony is the proud owner of two beagles, Nelson and Gracie Caruso, who are loyal and adorable. He is the president of the HTOWN men's lacrosse club, which competes throughout Texas in the southwest lacrosse association (SWLA).